Aus dem Max-Planck-Institut für Psychiatrie

Ärztliche Direktorin: Prof. Dr. Dr. Elisabeth Binder

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The role of the TGFβ signaling pathway in clinically non-functioning pituitary adenoma and the potential role of inhibitors of this pathway as a new target in pituitary tumor therapy

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Marie Céline van
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der Universität München

Berichterstatter:	Prof. Dr. Günter Stalla
Mitberichterstatter:	PD Dr. Walter Rachinger
	Prof. Dr. Eberhard Uhl
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. Ulrich Renner
Dekan:	Prof. Dr. med. Thomas Gudermann

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Table of Contents

Zusammenfassung **6**
Summary **10**
List of figures **13**
List of tables **14**
Abbreviations **15**

1. Introduction **18**
1.1 The pituitary gland and its function 18
1.1.1 Basic Anatomy of the pituitary gland 18
1.1.2 The neurohypophysis and the hypothalamic-pituitary axis (HPA) 19
1.1.3 The adenohypophysis 20
1.1.3.1 Distinct endocrine cellular lineages within the anterior pituitary gland 20
1.1.3.2 Folliculo-stellate cells 22
1.2 Pituitary tumors and tumorigenesis 22
1.2.1 Epidemiology and Nomenclature 22
1.2.2 Classification 23
1.2.3 Prolactinoma 28
1.2.4 Non- functioning Pituitary Adenoma 31
1.2.4.1 Definition and Overview 31
1.2.4.2 Epidemiology 32
1.2.4.3 Characteristic signs and symptoms 32
1.2.4.4 NFPA subtypes and WHO classification 33
1.2.4.5 Invasive and aggressive pituitary adenoma 37
1.2.5 Conventional treatment options for pituitary tumors 37
1.2.5.1 Prolactinoma therapeutic guidelines 37
1.2.5.2 Dopamine agonist resistant Prolactinoma 38
1.2.5.3 Treatment of hormone-secreting adenoma other than prolactinoma 38
1.2.5.4 Treatment of NFPA 39
1.2.5.5 Treatment of aggressive pituitary adenoma 41
1.3 The TGFβ signaling pathway 42
1.3.1 TGFβ-superfamily, structure and secretion pattern 42
1.3.2. TGFβ receptors 45
1.3.3 TGFβ-induced Smad signaling and alternate intracellular pathways 47

1.3.4 The TGFβ signaling pathway and tumorigenesis 50
1.3.5 The TGFß signaling pathway and VEGF 52
1.3.6 Differences in sex of the TGF signaling pathway mediated by dopamine and estradiol contributing to different pituitary tumor manifestations and outcome 53
1.3.7 The TGFß signaling pathway, EMT, fibrosis and DARP 55
1.3.8 The Role of the TGFß signaling pathway in NFPA 57
1.4 Aim of this Study 57

2. Materials and Methods 58
2.1 Chemicals 58
2.2.1 Culture media, buffer, diluents 58
2.2.2 Inhibitors of the TGFβ signaling pathway 58
2.2 Cell lines and primary cell cultures 60
2.2.1 Cell Culture of Mouse-Model Cell Lines TtT-GF and αT3-1 60
2.2.2 Primary Cell Cultures of Human Pituitary Adenoma Cells 63
2.3 Methods 65
2.3.1 Measurement of Cell Proliferation 65
2.3.1.1 ^{3}H-thymidine- Incorporation Assay 66
2.3.1.2 WST-1-Assay 67
2.3.2 Measurement of VEGF production 68
2.3.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Northern-blot 71
2.3.3.1 RNA isolation 71
2.3.3.2 Reverse Transcription 72
2.3.3.3 Polymerase Chain Reaction 72
2.3.3.4 Gel electrophoresis (Northern Blot) 72
2.5 Statistics 73

3. Results 74
3.1 Detection of the TGFβ signaling pathway in pituitary tumor cell lines 74
3.2 Effects of TGFβ isoforms and TGFβ inhibitors on the proliferation of mouse pituitary tumor cell lines 75
3.2.1 Proliferation studies with the WST-1-assay 75
3.2.2 Proliferation studies with the 3H-tymidine incorporation assay 76
3.3 Effects of TGFβ isoforms and TGFβ inhibitors on the proliferation of human nonfunctioning pituitary adenomas (NFPAs) 80

3.4 Effect of TGFß isoforms and inhibitors on the production of VEGF in αT3-1 and TtT/GF cells 84

4 Discussion 88
4.1 Discussion of the results 88
4.1.1 Expression of the TGFß system 88
4.1.2 Influence of TGFß and inhibitors on proliferation 89
4.1.3 Influence of TGFß and its inhibitors on VEGF-production 92
4.2 General discussion 94
4.2.1 Pituitary tumor development and EMT 94
4.2.2 VEGF and vascularization in pituitary tumors 97
4.3 Limitation of the study 98

References 100
Appendices 108
Danksagung (Acknowledgements) 109
Affidavit 110

Zusammenfassung

Das parenchymatöse Gewebe der Adenohypophyse besteht aus vielen verschiedenen endokrinen Zellen, welche unmittelbar nach den von den Zellen des Hypothalamus ausgehenden *releasing hormones* alle anderen endokrinen Organe in ihrer Aktivität steuern, mit wenigen Ausnahmen. Ungefähr 10-15% aller intrakraniellen Tumoren haben ihren Ursprung aus einer dieser Zellen und führen entsprechend häufig zunächst vor allem zu Symptomen, welche aus der Störung der hypothalamisch-hypophysären endokrinen Achse des jeweiligen Hormons resultieren. 30-40% aller Hypophysentumoren sind allerdings nicht hormon-sezernierend und werden entsprechend auch als „non-functioning"-Hypophysentumoren (engl. *Non-functioning pituitary adenoma*, NFPA) bezeichnet. Der Großteil der NFPAs stammen dabei von gonadotropin-sezernierenden Zellen ab. NFPA werden oft erst durch Masseneffekte symptomatisch, die endokrine Funktion der Adenohypophyse bleibt intakt, solange die hormon-produzierenden Zellen in ihrer endokrinen Funktion durch die Tumormasse nicht kompromittiert werden. Die operative Entfernung stellt die Erstlinientherapie für diesen Typ von Tumoren dar, solange keine Kontraindikationen für ein chirurgisches Vorgehen vorliegen. Aufgrund der zunehmenden Verdrängung extrasellärer Strukturen ist eine komplette Resektion des Tumors häufig herausfordernd, wenn nicht sogar unmöglich für den behandelnden Chirurgen. Mehrfache Operationen, neoadjuvante und adjuvante Bestrahlungen und manchmal auch Chemotherapien sind zur Behandlung der rezidivierenden Tumoren häufig notwendig. In der Konsequenz führen diese Tumoren, auch wenn sie selbst selten aggressiv sind, zu einer signifikanten Morbidität für den Patienten. Alternative pharmakologische Therapiekonzepte sind entsprechend notwendig, auch um eine maligne Transformation dieser Tumore zu verhindern.

Mitglieder der Transforming growth factor β (TGFβ)-Superfamilie, darunter die TGFβ-Isoformen TGFβ-1 und TGFβ-3 stellen bekannte Impulsgeber für das Wachstum von soliden epithelialen Tumoren dar, können dabei aber auch hemmend auf das Tumorwachstum einwirken. Initial hemmen sie die Bildung von Tumoren, während sie in fortgeschrittenen Stadien, vor allem nach dem Schritt der epithelialen-mesenchymalen Transition, die Tumorprogression fördern. Erst kürzlich wurden Inhibitoren der TGFβ-Isoformen und der intrazellulären Bestandteile der

Signalkaskade entwickelt, darunter der TGFβ-Rezeptor Suppressor SB431542, sowie SIS3, ein Inhibitor des TGFβ assoziierten intrazellulären Signalproteins Smad3.
In dieser Arbeit möchte ich den Einfluss der jeweiligen Inhibitoren auf das Wachstum der hormonell inaktiven hypophysären Tumorzellen ergründen, zunächst an zwei modelhaften Mauszelllinien: 1) den TtT-GF-Zellen als ein Model der Follikulostellatzellen, welche neben den hormonproduzierenden Zellen in der Hypophyse eher supportive und regulierende Funktionen einnehmen und eine Schlüsselrolle bei der Ausbildung der Tumormikroumgebung darstellen, 2) αT3-1-Zellen eine hypophysäre gonadotrope Mauszelllinie, vor dem Hintergrund, dass die Mehrheit der Non-functioning-Tumoren am ehesten von gonadotropen Zellen auszugehen scheint. Ich stimulierte diese Zellen mit TGFβ-1/-3, allein oder in Kombination mit den Inhibitoren SIS3 und SB431542, um dann die differente Proliferationsrate mittels des ^{3}H-thymidine Incorporation-Assays zu messen und zu vergleichen. Diese Ergebnisse verglich ich anschließend mit entsprechenden Experimenten des TGFβ-Signalwegs an einer kleinen Serie von 6 humanen NFPA-Primärzellkulturen. Außerdem untersuchte ich den Effekt der jeweils genutzten Inhibitoren des TGFβ-Signalwegs auf die Produktion des Zytokins VEGF (*Vascular endothelial growth factor*) in den oben genannten murinen Zelllinien, welches einen Schlüsselfaktor in der Tumorzellvaskularisation und entsprechend auch der Tumorexpansion darstellt. Aufgrund des limitierten Gewebematerials verzichtete ich auf entsprechende vergleichende Experimente bezüglich der VEGF-Produktion in den humanen Primärzellkulturen. Sowohl SB431542 als auch SIS3 hatten eine dosisabhängige, signifikante proliferationshemmende Wirkung sowohl auf TtT/GF als auch auf αT3-1 Zellen, sowohl mit als auch ohne externe Stimulation durch TGFβ- 1 oder TGFβ-3. Allerdings hatte sogar in hohen Konzentrationen die externe Stimulation der murinen Zelllinien mit den TGFβ-Isoformen TGFβ- 1/-3 keinen so hohen Einfluss auf die Proliferationsrate gezeigt, wie vorab erwartet. Der eher geringe stimulierende Effekt von TGFβ- 1 und TGFβ- 3 allein und der deutliche proliferationshemmende Einfluss der TGFβ-Inhibitoren, auch ohne externe Applikation von TGFβ, lassen vermuten, dass aus bislang noch unbekannten Gründen dieser Signalweg in schnell wachsenden TtT/GF- und αT3-1-Zellen intrinsisch und konstitutiv aktiviert ist. Mittels Northern Blot konnte ich zeigen, dass die mRNA nicht nur der TGFβ-Rezeptoren, sondern auch der intrazellulären SMAD-Signalproteine (Smad 2, 3 und 4) sowie die getesteten TGFβ-Isoformen (TGFβ-1,TGFβ-3) sowohl von TtT/GF- als auch von αT3-1-Zellen exprimiert werden.

Das legt nahe, dass TGFβ von diesen Zelllinien selbst sezerniert wird und die Aktivierung dieses Signalwegs auch auf autokrinem Wege entsprechend Einfluss auf Wachstum und Funktion von αT3-1- und TtT/GF-Zellen nimmt. Dies lässt eine gewisse Bedeutung dieses Signalweges für die verschiedenen hypophysären Zelltypen vermuten.

In jedem Fall besitzen die TGFβ–Inhibitoren SIS3 und SB431542 damit eine potente proliferationshemmende Wirkung auf diese Zelllinien. In den Experimenten mit den Primärzellkulturen, gewonnen aus 6 Tumorresektaten von NFPAs, stellte sich ein differenzierteres Bild dar. In einer Primärzellkultur führte TGFβ-1 zu einer signifikanten Hemmung des Zellwachstums, in zwei anderen führte es eher zu einer leichten, nicht signifikanten Stimulation des Zellwachstums, in drei weiteren Tumorzellproben konnte hingegen ein signifikanter proliferationssteigender Effekt gezeigt werden. Der Effekt von TGFβ–3 konnte nur in zwei Adenom-Primärzellkulturen getestet werden und in keiner von beiden konnte ein stimulierender oder hemmender Effekt durch die Applikation von TGFβ–3 detektiert werden. In den meisten Fällen kam es auch unter alleiniger Applikation der untersuchten TGFβ–Inhibitoren zu einer Abnahme der Proliferation der asservierten Adenom-Zellen. In der Zellkultur des Tumors NFPA1, wo TGFβ–1 einen antiproliferativen Effekt aufwies, führte die kombinierte Gabe von TGFβ-1 und den getesteten Inhibitoren zu einer gesteigerten Proliferation im Vergleich zu der Basisproliferationsrate ohne externen Stimulus. In den Zellkulturen von Tumor NFPA1, wo TGFβ–1 die Zellproliferation hemmte, war bei kombinierter Gabe von TGFβ–1 und den Inhibitoren insgesamt ein wachstumsstimulierender Effekt zu beobachten. Die singuläre Gabe von SB431542 führte zu einem kleinen Anstieg der Proliferationsrate, die durch die Kombination mit TGFβ–1 noch potenziert wurde. SIS3 hingegen wirkte für sich genommen wachstumshemmend, ein Effekt der bei höherer Dosierung allerdings wieder abnahm, was zu den Ergebnissen der kombinierten Applikation von SIS3 und TGFβ–1 passt. In den anderen NFPA-Primärzellkulturen in denen TGFβ–1 wachstumsstimulierend wirkte, führte die alleinige oder kombinierte Behandlung mit den getesteten Inhibitoren zu einer signifikanten Hemmung der Proliferationsrate. Obwohl TGFβ–3 allein keinen Effekt auf die Proliferationsrate zeigte, führte die kombinierte Applikation von TGFβ–3 mit den getesteten Inhibitoren SIS3 und SB431542 zu einem Ausschalten der wachstumshemmenden Effekte der TGFβ– Inhibitoren. Wie in den murinen Tumorzelllinien konnte auch ein meist

hemmender Einfluss auf das Zellwachstum durch die Inhibitoren ohne die externe Applikation von TGFβ bei den humanen Tumoren beobachtet werden. Die Ergebnisse mit den humanen NFPA-Primärzellkulturen lassen vermuten, dass die jeweiligen TGFβ–Inhibitoren und involvierten intrazellulären Signalkaskaden die Proliferation und entsprechend Expansion der NFPAs nur unter bestimmten Konditionen hemmen, vermutlich eher in fortgeschrittenen Tumorstadien. Es wäre perspektivisch interessant, das Patienten-Outcome und klinische Verhalten dieser Tumoren, aus denen unsere oder zukünftige Proben stammen, mit den experimentellen Ergebnissen zu vergleichen und molekulare und/ oder klinische prädiktive Marker hinsichtlich einer potentiellen Wirkung dieser Inhibitoren zu finden, bezüglich des Ansprechens der Tumoren auf die Behandlung mit TGFβ und den jeweiligen Inhibitoren der Signalkaskade.

In den Experimenten zur VEGF-Sekretion führten sowohl TGFβ-1 als auch -3 zu einer gesteigerten Produktion dieses provaskulären Faktors in TtT/GF- und αT3-1-Zellen. Die getesteten Inhibitoren führten zu einer signifikanten Hemmung sowohl der TGFβ-induzierten als auch der basalen VEGF-Sekretion in den untersuchten Zelllinien.

Auch wenn aufgrund limitierter Menge an Gewebeproben keine entsprechenden Experimente für die humanen NFPA-Primärzellkulturen erfolgen konnten, ist es wahrscheinlich, dass SB431542 und SIS3 auch die VEGF-Produktion in NFPAs und damit die Bildung dieser Tumoren durch den hemmenden Einfluss auf die Tumorvaskularisation unterdrücken.

Zusammengefasst konnte in diesen Experimenten gezeigt werden, dass die TGFβ-Inhibitoren potentiell die Progression von NFPAs verlangsamen, wenn nicht sogar blockieren können, insbesondere wenn es bereits zur epithelialen mesenchymalen Transition (EMT) gekommen ist und die Tumoren einen aggressiveren Phänotyp erlangt haben. Entsprechend kann es sich bei den ausgewählten TGFβ-Inhibitoren um interessante Werkzeuge neuer pharmakologischer Behandlungskonzepte für diese Tumorentität handeln. In jedem Fall ist es möglich, durch die spezifischen Inhibitoren der Bestandteile dieses komplexen Signalwegs zu einem vertieften Verständnis der verschiedenen jeweiligen Elemente in der TGFβ-Signalkaskade und ihrer Alterationen im Rahmen der Tumorprogression zu erreichen, was zur Entdeckung möglicher neuer gezielter Therapieansätze führen kann. Dazu werden allerdings noch weitere Studien und experimentelle Designs notwendig sein.

Summary

The parenchyme of the anterior pituitary gland consists of various distinct endocrine cells which second to the hypothalamic -releasing hormones regulates all other endocrine organs with few exceptions. About 10-15% of all intracranial tumors derive from one of those endocrine cells, leading to symptoms mainly due to the disruption of the hypothalamic-hypophysial-endocrine axis. Nonfunctioning pituitary adenomas (NFPAs) account for about 30 to 40 % of all pituitary tumors and the majority of them originate from gonadotroph pituitary cells.

NFPAs typically become symptomatic due to mass effects, the endocrine function of the adenohypophysis remains intact as long as the functioning hormone-secreting cells are still not compromised due to tumor volume. Surgical removal is the first line of therapy, if no contraindications against surgery exist. However, as they start to invade neighboring extrasellar structures, complete resection is often challenging if not impossible for the surgeon. Repeated surgeries neoadjuvant and adjuvant radiotherapy and sometimes chemotherapy are often necessary to treat recurrent NFPA. Consequently, although they are rarely aggressive, they lead to a significant morbidity for the patient. Therefore, alternate pharmacological treatment concepts for NFPA, are needed, also to prevent the transformation of these tumors to more aggressive ones.

Members of the transforming growth factor β (TGFβ) protein family, among them TGFβ-1 and TGFβ-3 isoforms, play a contrary role in the process of tumor formation. They are well-known as potential promoters of the growth of solid epithelial tumors, though can also exhibit tumor suppressive effects. Initially they block the development of tumors, whereas in advanced stage, in particular after epithelial tumors had undergone epithelial mesenchymal transition (EMT), they stimulate tumor progression. Only recently, inhibitors of the action of TGFβ isoforms have been developed, among them the TGFβ receptor suppressor SB431542, and SIS3, an inhibitor of the TGFβ induced intracellular signaling protein Smad3. In the present thesis I want to study the influence of the TGFβ inhibitors on the growth of hormonally inactive pituitary tumors, using two hormonally inactive murine pituitary tumor cell lines: (1) TtT/GF cells as a model of folliculo-stellate cells, which show supportive regulatory within the pituitary gland and which play a key role in the formation of tumor microenvironment. (2) Gonadotroph-like αT3-1 cells, as the majority of NFPA seem to derive from gonadotropin-producing cells. Stimulating these cells with TGFβ-1/-3, alone or in

combination with the inhibitors SIS3 and SB431542 I measured the difference in cell viability using the ^{3}H-thymidine incorporation assay. I compared these results with those obtained with corresponding experiments on TGFβ-signaling in a small series of 6 human NFPA-samples in primary cell culture. I further studied the effect of the respective chosen TGFβ inhibitors on the production of vascular endothelial growth factor (VEGF), a key factor of intratumoral vessel formation and thus tumor expansion, in the two murine cell lines. Due to the limited tissue material available, I did omit from VEGF-experiments in human NFPA cell cultures.

Both SB431542 and SIS3 significantly inhibited the proliferation of TtT/GF and αT3-1 cells in a dose dependent manner both in the absence and presence of TGFβ-1/-3. However, even at high concentrations the external stimulation with the TGFβ isoforms, the stimulatory influence on the proliferation of the murine cell lines and primary cell cultures was not as high as expected beforehand. The small growth stimulatory effect of TGFβ- 1 and TGFβ- 3 alone and the clearly visible suppressive action of the inhibitors on the basal growth of the cell lines without external application of TGFβ suggests, that for reasons that are so far not known, the TGFβ signaling system is intrinsically and constitutively activated in rapidly growing TtT/GF and αT3-1 cells. Using northern blot analysis I could show the mRNA-expression of not only the TGFβ receptors, but also the subsequent signaling proteins (Smad 2,3 and 4) and the tested TGFβ isoforms (TGFβ-1,TGFβ-3) in both TtT/GF and αT3-1 cells.

This suggests that TGFβ may be produced by the cell lines and that the activation of this pathway may thus affect growth and function of αT3-1 and TtT/GF cells in an autocrine manner as well. This stresses the potential importance of this signaling pathway in these distinct pituitary cell-types.

Either way, the two inhibitors SIS3 and SB431542 seem like potent suppressors of the proliferation of the cell lines. The situation is different in primary cell cultures derived from 6 human NFPAs. Here TGFβ-1 significantly inhibited the proliferation in one tumor cell culture, slightly but not significantly stimulated cell growth in 2 cases, and significantly stimulated cell proliferation in 3 tumor cell cultures. The role of TGFβ−3 could be tested only in 2 adenoma cell cultures and in none of them a stimulatory or inhibitory effect on cell proliferation could be detected. In most cases the inhibitors suppressed the proliferation of the adenoma cells already in the absence of TGFβ isoforms. In the cell culture of NFPA1, in which TGFβ−1 inhibited the cell proliferation, the combined application of TGFβ−1 and the inhibitors led to a growth stimulatory

effect. SB431542 alone led to a small spurt in tumor growth in the NFPA1-sample, that was potentiated in combination with TGFβ−1, whereas SIS3 suppressed cell growth – this effect decreased though at the higher dosage, which corresponded with the results of the combined treatment of SIS3 with TGFβ−1. In the remaining tumor cell cultures, in which TGFβ−1 had growth-stimulatory effects, the treatment with the inhibitors combined or alone led to a significant reduction of proliferation rate. Although TGFβ−3 alone had no effect on cell proliferation, the combined application of TGFβ−3 and the inhibitors abolished the growth suppressive effects of the inhibitors. As in the murine tumor cell lines, a suppressive effect of the two inhibitors was also seen in the absence of external TGFβ-1. The results obtained from the limited number of tumors suggest that TGFβ inhibitors may suppress the proliferation and thus the expansion of NFPAs only under certain conditions, probably in advanced stages of tumor development. It would be particularly interesting to compare the patient outcome and clinical behavior of the tumors from which our or new samples derive with these experimental results and to find molecular or clinical markers predicting the response towards TGFβ-isoforms and the individual inhibitors of the corresponding pathway. Regarding VEGF secretion, both TGFβ-1 and -3 stimulated the production of this angiogenic factor in TtT/GF and αT3-1 cells. The tested inhibitors significantly inhibited both TGFβ-promoted and basal VEGF secretion in the cell lines. Although no corresponding studies could be done in human NFPAs due to lack of tissue, SB431542 and SIS3 may probably also suppress the VEGF production in NFPAs and may therefore suppress the development of these tumors by inhibiting tumor vascularization.

Taken together, the data suggest that TGFβ inhibitors may slow down or block the progression of NFPAs, in particular those that have passed epithelial mesenchymal - transition (EMT) and have developed a more aggressive phenotype.

In any case, this research suggests that with the recently developed specific inhibitors, SIS3 and SB431542 it is possible to reach a further in-depth understanding of the various respective elements involved in the TGFβ signaling cascade and their alterations during tumor progression, leading to possible new targeted therapeutic options. This, however, implies further studies and experimental designs than this small study encompasses.

List of figures

Figure 1: Simplified scheme of the differentiation of the individual anterior pituitary cell types originating from the neuroectoderm of Rathke´s pouch. 21

Figure 2: Secretion of gonadotroph free subunits by clinically non-functioning gonadotropinoma, the most common subtype of NFPA. 35

Figure 3: Schematic illustration of the percentage of clinically non-functioning pituitary adenoma identified by solely immunohistochemistry for anterior pituitary hormones alone (left) and with the inclusion of transcription factors for diagnosis (right) (Drummond et al., 2018 [2]). 36

Figure 4: Systematic flowchart of clinical procedure guidelines for incidentally found pituitary lesions [8, 9] 41

Figure 5: Schematic illustration of the type-I and type-II-receptors and the selective binding of the respective members of the TGFβ-superfamily [3] 46

Figure 6: Schematic illustration of the Smad- dependent and Smad independent TGFβ induced intracellular pathways adapted from Xia et al., 2015 [4]. .. 50

Figure 7: Schematic illustration of the Smad- dependent and Smad independent TGFβ induced epithelial-to mesenchymal transition and the genes/ transcription factors involved, adapted from Katsuno et al., 2013 [5] 56

Figure 8: Neubauer chamber for cell counting. 63

Figure 9: Principle design of Sandwich-ELISA 70

Figure 10: Detection of mRNA of TGF-β isoforms, TGF-β receptors and SMAD signaling proteins. 74

Figure 11: Representative results from proliferation experiments with the TtT/GF cell line using the WST-1 assay. 76

Figure 12: Effect of TGFβ–1 and TGFβ inhibitors alone or in combination on 3H-thymidine incorporation of αT3-1 cells.. 78

Figure 13: Effect of TGFβ–1 and TGFβ inhibitors alone or in combination on 3H-thymidine incorporation of TtT/GF cells. 79

Figure 14: Effect of TGFβ isoforms and inhibitors in primary cell culture of NFPA1. rowth. 81

Figure 15: Effect of TGFβ isoforms and inhibitors in primary cell culture of NFPA2.. 82

Figure 16: Effect of TGFβ–1 and inhibitors in 4 primary cell cultures of NFPAs. 83

Figure 17: Effect of TGFβ–1 and TGFβ inhibitors alone or in combination on VEGF production of αT3-1 cells. 85

Figure 18: Effect of TGFβ–1 and TGFβ inhibitors alone or in combination on the VEGF production of TtT/GF cells. 86

List of tables

Table 1: Pituitary adenoma subtypes 26

Table 2: Morphometric classification and the terms Invasiveness, Aggressiveness and Carcinoma in regard to pituitary tumors:. 28

Table 3: IHC for PIT1 (pituitary-specific positive transcription factor 1; coded by the POU class 1 homeobox 1 gene), SF1, TPIT (coded by the T-box transcription factor 19 gene), and estrogen receptor- α (ER α) allowed for the identification of mutually exclusive lineage-specific markers in 95% of the cases, reducing the prevalence of the null cell adenoma subtype to ~1% [1, 2]..... 34

Table 4: Overview of the TGFβ-superfamily..... 43

Table 5: Culture media used 58

Table 6: Overview about established methods for the determination of cell proliferation and viability (derived from Yadav et al. 2001 [163]..... 66

Table 7: Primers used for the detection of RNA of TGFß, TGFß receptors and Smad signaling components..... 73

Table 8: Effect of TGFβ inhibitors on the proliferation of TGFβ–3 treated αT3-1 and TtT/GF cells 80

Table 9: Effect of TGFβ inhibitors on the TGFβ-3 stimulated VEGF production in αT3-1 and TtT/GF cells 87

Abbreviations

A	
ACTH	Adrenocorticotropic hormone
Akt/PKB	Protein-kinase B, syn. RAC-serine/threonine-protein kinase
ALK	Anaplastic lymphoma kinase, syn. ALK-tyrosine kinase receptor, cluster of differentiation 246
AMH	Anti- Mueller hormone
ANOVA	Analysis of variance
B	
BMP	Bone morphogenetic protein
BSA	Bovine-serum albumin
C	
CAF	Cancer associated fibroblasts
CDKN2A/p16	Cyclin-dependent kinase Inhibitor 2A, protein 16
CNS	Central nervous system
CRM-1	chromosome region maintenance 1
CTH	corticotropic hormone
D	
DA	dopamine agonist
DARP	dopamine agonist resistant prolactinoma
DEPC	0.1% diethylpyrocarbonate (DEPC)
DNA	Desoxyribonucleic-acid
dNTP	Desoxy-nucleoside-triphosphate
DPC4	deleted in pancreatic carcinoma locus 4
Drd2	dopamine type 2 receptor, dopamine-receptor D2
DTT	Dithiothreitol
E	
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epithelial growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	epithelial-mesenchymal transition
EndMT	Endothelial-mesenchymal transition
ER	endoplasmatic reticulum
ER α	Estrogen-receptor α
ERK	Extracellular signal-regulated kinase
F	
FAD(H)	flavin adenine dinucleotide (FAD)-H (hydrogen)
FMN	flavin-mononuleotide
FS- cells	folliculo-stellate cells
FSH	follicle stimulating hormone
FPA	Functioning pituitary adenoma
FYVE	Protein domain consisting of Fab1 (yeast orthologue of PIKfyve, YOTB, Vac1 (vesicle transport protein) and EEA1. It binds phosphatidyl-inositol-3-phosphate
G	
GADD-43	growth arrest and DNA damage protein 43
GADPH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GATA22	GATA-22 transcription factor
GDF	growth differentiation factor
GDNF	Glial-derived neurotrophic factor
GH	growth hormone, somatotropin (see STH)
GnRH	Gonadotropine releasing hormone
GP	Glycoprotein

H	
hCG	Human chorion gonadotropine
HdB+-buffer	High density buffer
HDM2	Human double minute 2 protein, a ubiquitin-3-ligase that binds to p53
HIF1	hypoxia inducable factor 1
HPA	hypothalamic pituitary neuroendocrine axis
HRP (Streptavidin-HRP)	Horse-radish peroxidase
I	
IL-6	Interleukin- 6
I-Smad	inhibitory Smad
J	
JNK	Janus kinase
L	
LAP	latency associated peptide
LH	luteinizing hormone
LTBP	latency binding protein
M	
MAPK	mitogen-activated protein kinase
MH1	MAD homology 1
MMP	Matrix-Metallo-proteinase
mTOR	Mammalian or mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
N	
NADH	Nicotinamide adenine dinucleotide
NFMA	Non- functioning pituitary macroadenoma
NFPA	Non- functioning pituitary adenoma
P	
PBD	PP1c-binding domain
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDAC	Pancreatic-ductal-adenocarcinoma
PRL	prolactin, syn. luteotropic hormone
PI3K	Phosphoinositide 3-kinase
P-Smad	phosphorylated Smad or in another context, pathway restricted Smads
POMC	Proopiomelanocortin
PP1	Protein-phosphatase 1
PPAR-γ	Peroxisome proliferator activated receptor γ
PVN	paraventricular nuclei
R	
RIA	Radioimmunoassay
RNA	ribonucleic acid
R-Smad	Receptor- Smad
S	
SARA	Smad-anchor for receptor activation
SBD	Smad-binding domain
SCA	Silent corticotroph adenoma
SF-1	Steroidogenic factor 1
Smad	acronym from the fusion of Caenorhabditis elegans Sma genes and the Drosophila Mad, Mothers against decapentaplegic) proteins to transduce signals
Smurf 1	Smad ubiquitylation regulatory factor 1
SON	supraoptic nuclei

SSXS motif	Ser-SerVal/Met-Ser sequence (a characteristic peptide-sequence/motif on the C-terminsl ends of receptor-Smads
STH	Somatotropin, somatotropic hormone also see GH
α-SU	alpha subunit
T	
TAK1	TGFβ activated kinase 1
TCA	trichlor-acetic acid
[^{3}H]-TdR	Tritium radiolabeled thymidine
TEF	Thyreotroph-embryonic factor
TGFβ	Transforming-growth factor β -1, -2, -3
TGFβRI	TGFβ- receptor I
TGFβRII	TGFβ- Receptor II
TLP	TRAP-1-like protein (also see TRAP)
tPIT	t-box- pituitary transcription factor
TSH	Thyreoid-stimulating hormone
V	
VEGF	Vascular endothelial growth factor (mVEGF – mouse VEGF, hVEGF human VEGF)
W	
WIF1	Wnt inhibitory factor 1
Wnt	Wingless integration 1 (signaling pathway)
WST-1	Water-soluble-tetrazolium 1

1. Introduction

1.1 The pituitary gland and its function

The pituitary gland represents a complex organ acting as an essential link in between the Central nervous system and the function of peripheral organs by regulation of the endocrine axis. It consists of two functionally and evolutionarily distinct lobes: The Adenohypophysis, anatomically referred to as the anterior pituitary gland, and the neurohypophysis or posterior pituitary gland [1, 2]. The posterior lobe contains nerve endings of the hypothalamus, an essential part of the forebrain [3]. It is connected to the anterior lobe via a system of blood vessels, commonly referred to as the hypophysial portal system. By this connection the so called hypothalamic- pituitary neuroendocrine axis (HPA) is formed. The pituitary gland therefore is an essential organ, occurring in all mammalian brains [1], concerning many medical disciplines such as endocrinology, gynecology, neurology, and internal medicine. Although pituitary tumors are generally rare, symptoms associated with it might be of concern for physicians in all medical disciplines.

1.1.1 Basic Anatomy of the pituitary gland

The hypophysis lies in the *sella turcica*, a small saddle-shaped depression in the body of the sphenoid bone at the base of the skull [4]. The height of the pituitary gland ranges between 3-9mm in the saggital plan [4, 5]. It consists of two lobes, the anterior lobe, which constitutes around two-third of total pituitary volume [3], and the posterior lobe. Anterior and posterior lobes are connected at the so-called *pars intermedia*. The posterior lobe is also connected to the forebrain via a small infundibulum, which is sometimes referred to as the pituitary stalk. As the gland lies within the skull it is surrounded by Dura. Directly above the hypophysis the Dura forms a thickened membrane, the *diaphragma sellae*, which extends from the dorsum sellae to the tuberculum sellae and the medial dural walls of the cavernous sinuses [4]. It contains a small hole through which the infundibulum of the posterior lobe is connected to the forebrain [4]. About 5 to 10 mm above the diaphragma sellae, on the other hand lies the optic chiasm [3]. Laterally to the sella turcica and pituitary gland lie the intracavernous sinuses [4] which contain cranial nerves (CN) 3,4,6, and the first and second branches of the fifth cranial nerve (V1 and V2),[3]. Due to the close relation of the pituitary gland to those structures, mass effects in this region lead to

ophthalmological and neurological symptoms accordingly. Bitemporal heteronyme hemianopsia, also referred to as “the chiasmatic syndrome” is almost pathognomonic for tumors of the sella region, which are mainly caused by hypophysial adenomas. General visual deficits, scotomas, and other fundus pathologies often lead the patient to the ophthalmologist ([4] p.179). Cranial nerve dysfunction due to pituitary tumor occur in approximately 5-17% of pituitary tumor patients [6-8], mainly involving III,IV,V CN in that order [6-8]. Thus extraocular muscle dysfunction or ipsilateral facial pain can occur as a presenting symptom of pituitary lesions [3]. Although in most cases hypophysial lesions manifest with endocrine disruptions as a leading symptom, it is important to remember the close anatomical relations of the pituitary gland to the other structures in the skull, especially concerning patients with silent nonfunctioning pituitary adenoma.

1.1.2 The neurohypophysis and the hypothalamic-pituitary axis (HPA)

The posterior pituitary gland or neurohypophysis, though significantly smaller than its anterior counterpart, represents the essential connecting link in between the CNS and the endocrine system. In contrast to the adenohypophysis the neurohypophysis directly derives from the midline portion of the anterior neural ridge (ANR) as part of the forebrain [1]. The neurohypophysis can be divided into three sections: The neural lobe or pars nervosa (sometimes referred to as infundibular process), the median eminence of the hypothalamic tuber cinerum and the infundibular stem [8]. Histologically, the posterior lobe mainly consists of the axonal terminals of neurons from the supraoptic nuclei (SON) and paraventricular nuclei (PVN) of the hypothalamus. They are surrounded by pituicytes and other glial cells that might also be involved in the finetuning of axonal hormonal release [2]. Within the posterior gland at the median eminence neuro secretive peptide hormones are released. Some of these are called *releasing hormones*, as they trigger the release of their corresponding hormonal counterpart of the respective endocrine cell in the adenohypophysis. They reach those cells via the hypophysial portal system, a connecting system of blood vessels formed by anastomoses from branches of the anterior and posterior hypophysial arteries. Other important hormones that are released within the posterior pituitary are Oxytocin, Vasopressin, Somatostatin and Dopamine.

1.1.3 The adenohypophysis

The anterior pituitary gland serves for the secretion of the stimulating hormones for the respective glands in the body. It derives from the Rathke`s Pouch, an invagination of the oral ectoderm. The development and differentiation of the diverse cell-types is primarily induced by the ventral diencephalon [1]. The anterior pituitary can be divided into three anatomical sections: Pars tuberalis (syn. infundibularis), pars intermedia and pars distalis. The pars distalis is by far the biggest portion of the adenohypophysis, making up around 80% of adenohypophysial volume [2]. On a cellular level 6 main, mature endocrine cell types can be found: Corticotropes (POMC, ACTH), thyreotropes (TSH), gonadotropes (LH, FSH), somatotropes (GH), lactotropes (PRL) and mammosomatotropes (GH, PRL) [9]. The release of the respective hormones by those cells is regulated by hypothalamic hormones, brought to them through the portal circulation system [9], which was mentioned before. Still, the trans-/ paracellular mechanisms leading to synchronized networks and secretion clusters of the target cells is not thoroughly understood [2]. Another common adenohypophysial, but nonhormonal cell type, which might play a role in building up those networks is the folliculo-stellate cell (FS). Folliculo-stellate cells make up approximately 5-10% of total pituitary cells [9]. Increasing evidence suggests an important role for these cells in regulating the function, intercellular communication and immune-endocrine-crosstalk of the endocrine pituitary cells [2]. Also, FS-cells are able to phagocyte cell debris. They express GFAP and S100β, two common markers of astrocytes. Therefore, a relation to microglia/astrocyte- like cells is commonly assumed [2].

1.1.3.1 Distinct endocrine cellular lineages within the anterior pituitary gland

The differentiation of the various respective hormone-secreting cell-types within the pituitary gland is regulated by lineage specific transcription-factors. By immunohistochemistry analysis, these transcription-factors can be measured. Combined with the measurement of the hormones secreted by those cells, the cells can be more clearly assigned to a specific lineage. By this, clinically silent pituitary adenoma (SPA), which do not produce hormones that could be measured by immunohistochemistry (IHC) can now be clearly assigned to a specific cellular lineage [10-12]. The detailed pathways of adenohypophysial cytodifferentiation, transcription factors implicated in the development of the respective hormone-secreting cell-type are shown in figure 1.

In short, the three pathways originating from the Rathke´s pouch stem cell during embryogenesis are [10].

1) Corticotrophs determined by the t-box- pituitary transcription factor (Tpit),
2) Somatotrophs/mammosomatotrophs/lactotrophs/thyrotrophs determined by pituitary transcription factor (Pit-1)
3) Gonadotrophs determined by steroidogenic Factor-1 (SF-1) and/ or GATA-2 in the presence of estrogen receptor α (ER α)

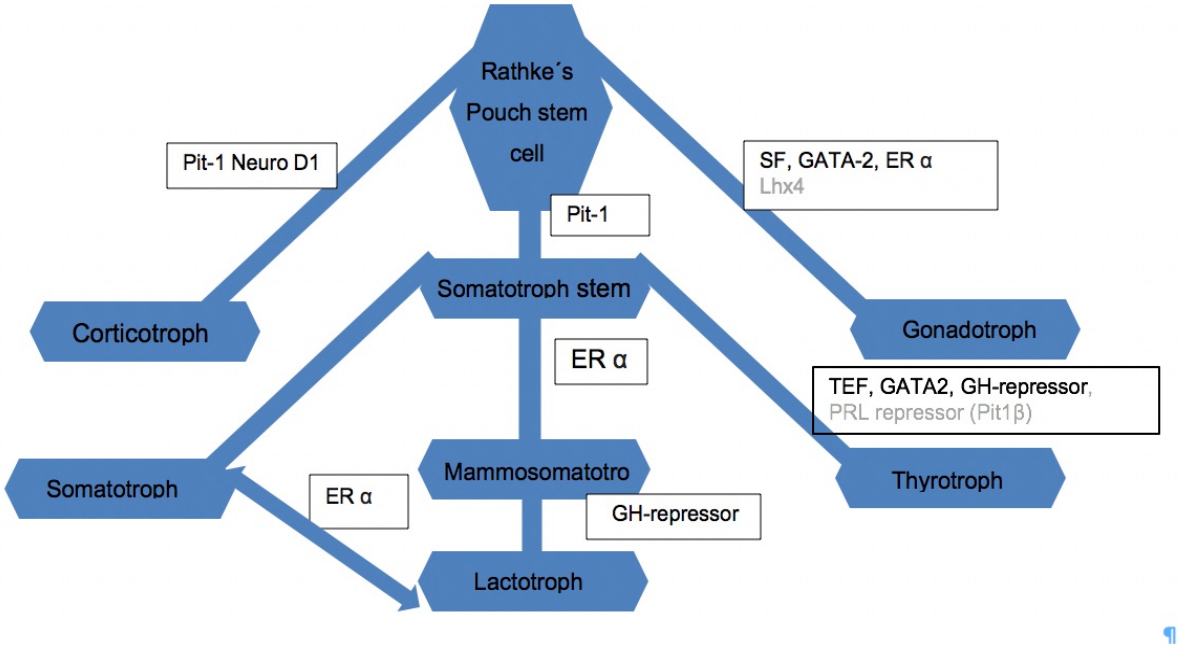


Figure 1: Simplified scheme of the differentiation of the individual anterior pituitary cell types originating from the neuroectoderm of Rathke´s pouch. The first molecular determinants that occur are that of the corticotroph lineage, Tpit (t-box pituitary transcription factor) and NeuroD1/β2. Pit-1 expression designates a somatotroph stem cell, which, in the absence of other transcription factor retains somatotroph morphology and function. Expression of estrogen receptor (ER) enhances PRL expression in mammosomatotrophs. The mammosomatortroph is at the center of a 3-way fluctuation indicated by 2-directional arrows. These cells transdifferentiate physiologically. A growth-hormone (GH) repressor is implicated in silencing GH transcriptions to allow the development of mature lactotrophs. Another cell-type deriving from the somatotroph stem cell is the thyreotrope cell. The determing transcription factors involved are TEF (thyreotroph embryonic factor) and GATA-2. Again, a putative GH-repressor is implicated in this differentiation pathway as well, silencing GH transcription. PRL repression may rely on Pit1β. The third main pathway is that of the gonadotroph lineage, dictated by SF-1 (steroidogenic factor 1) and GATA-2, which, in conjunction with ER and Lhx-4 determine gonadotroph differentiation and gonadotropine gene transcription [10]. All transcription-factors illustrated above, except of Pit1β and Lhx4, are taken into account for the WHO subclassification of non-functioning, potentially silent pituitary adenoma [11].

The individual transcription factors have been included in the last modification of the WHO classification 2017 of non-functioning pituitary adenoma (NFPA), which are the focus of this work.

1.1.3.2 Folliculo-stellate cells

The folliculo-stellate cell was first described by Rinehart and Farquhar in 1953, based on its appearance (agranular and stellate), its location around follicles and positive immunostaining for S100-beta (Rinehart and Farquhar 1953, Nakajima 1980, cited by [13]). FS-cells make up around 5-10% of anterior pituitary cells (Rinehart and Farquhar 1953). Folliculo-stellate cells seem to play a major role in the complex regulation of various endocrine functions of the pituitary gland as a mediating cell type. Gap junctions between FS cells allow synchronized excitability and long-distance communication throughout the pituitary via propagated Ca^{2+} currents in between FS cells [13]. Works of various authors suggest a mediator function of FS-cells in between the immune and endocrine system [13]. Furthermore, various studies have hinted that these cells are able to transdifferentiate, which raises the possibility that these cells do also represent a pituitary stem cell population [14]. Besides, folliculo-stellate cells are the only cells in the healthy pituitary gland that produce VEGF [15] , as well as other angiogenesis related substances such as basic fibroblast growth factor, leukemia inhibitory factor and Interleukin-6 [15]. This suggests a key role of the folliculo-stellate cell in the regulation of angiogenesis and vascular permeability within the pituitary gland [15].

Overall, it can be said that the anterior pituitary gland is an organ with a very heterogeneous parenchyma in which interactions of various different endocrine as well as non-endocrine cell- types is complexly regulated.

1.2 Pituitary tumors and tumorigenesis

1.2.1 Epidemiology and Nomenclature

Around 8-15% of all brain tumors are pituitary tumors, stemming from the anterior pituitary gland [8]. Adenohypophyseal tumors are usually benign, well-differentiated neoplasms, whose clinical relevance is usually restricted to the endocrine disruption depending on the affected cell-type. Experts therefore often summarized. The nomenclature of adenohypophyseal tumors have recently been under debate. Still, the recent WHO classification published in 2017 maintained the term “adenoma” although a sizeable proportion of these tumors shows invasive behavior towards surrounding structures and is not curable by standard treatment options [16]. A simplistic distinction in between adenoma and carcinoma is therefore misleading and neglecting the highly

variable impact of these tumors on patients [10]. This was criticized by many authors such as Trouillas et al. [16]. Consequently, in 2017, the International Pituitary Pathology Club proposed a change in terminology, speaking of Pituitary neuroendocrine neoplasms (PitNET), consistent with the terminology of other neuroendocrine neoplasms [10]. This shall reflect more closely the variability of these tumors and their behavior [16].

PitNETs or pituitary adenoma are still considered rare though increasing evidence suggests that prevalence rates might be as high as 1 in 1000 of the general population [7, 17, 18]. A large metaanalysis by Ezzat et al. from 2004 estimated the overall prevalence rate of pituitary adenoma to be 16,7% [19]. For the estimation of prevalence rate authors included both radiographic studies using MRI as well as postmortem histological autopsy studies of the pituitary gland [19].

They suggested though that, for one, small pituitary lesions might not be detected on MRI scans and secondly many patients remain undetected for lack of specific symptoms [19]. They further questioned incidentally found pituitary lesions to be clinically insignificant [19]. For instance, Watson et al. showed that in approximately 40% of cases with clinically relevant Cushing´s disease resulting from pituitary adenoma, the tumor lesions remain undetected even by sensitive MRI scanning techniques [19].

1.2.2 Classification

As the anterior pituitary gland consist of various different cell types, pituitary tumor patients can principally show a broad range of different symptoms, such as endocrine abnormalities or neurological deficits resulting from mass effects of the tumors ([8, 20]. They can be distinguished/ classified based on different parameters such as:

- Microadenoma/-carcinoma vs. Macroadenoma and Giant adenoma based on tumor volume
- Secreting tumors vs. silent tumors (functioning vs. non- functioning PAs)
- Benign adenoma, invasive and/or aggressive adenoma and carcinoma based on molecular features, therapy responsiveness and metastasis.
- Resistant/ responsive tumors to established treatment options

Tumors below a size of 10 mm are called microadenoma, those above are called macroadenoma [19, 21]. Adenoma with a size above 40 mm are also called giant adenoma [22]. For a more detailed description of the term, see table 2.

Pituitary tumors are usually monoclonal and show hormonal hypersecretion, based on the involved endocrine cell-type, independent of the HPA. The main secreting pituitary adenomas are prolactinoma, corticotropinoma and somatotropinoma, which due to their hormonal hypersecretion pattern independent of the HPA lead to characteristic clinical syndromes, such as Cushings Syndrome (CTH), or acromegaly (STH). As such they are clinically referred to as functional pituitary adenoma. For a detailed description of the characteristic symptoms of the respective adenoma subtype, see table 1. “Non-functioning pituitary adenoma” is primarily a clinical diagnostic term. It must further be distinguished in between *silent* pituitary adenoma and *clinically silent* pituitary tumors. In silent (but not *clinically* silent) adenoma hormone excess can just be proven immunohistochemically. Sometimes, clinically silent pituitary adenoma show immunohistochemical and additional biochemical evidence (elevated serum levels of the respective hormones) but no clinical evidence of hormone excess [17]. For instance, elevated serum levels of FSH could be shown in some “non-functioning” gonadotropinomas in vitro and in vivo [12, 23]. As the additional measurement of transcription factors of the respective lineages with IHC analysis became more established, NFPA could be further classified (also see Chapter NFPA).

Still, non-functioning pituitary adenoma do typically not present themselves with hormonal excess and the associated syndromes. They are either found as incidental findings on MRI [19, 20] or are being recognized by symptoms related to mass effects (since 67-90% of them are macroadenoma [6, 24-26]), by visual impairment, hypopituitarism [7, 18, 25, 27] and Diabetes insipidus [28].

By the old definition of the WHO from 2004, pituitary tumors were classified as *“atypical”* if their MIB-1 (Ki-67) proliferative index was above 3% and the tumors show significantly increased p53 immunoreactivity and mitotic activity [5, 17]. Up to 15% of resected pituitary adenoma are classified as aggressive pituitary adenoma [29]. These atypical adenomas were thought to behave particularly aggressive However, prognostic significance could not be established despite intensive research on that topic over the last decade [10]. Therefore, the term atypical adenoma was left in the last WHO classification in 2017. There is still some ambiguity for the term “aggressive adenoma” – clinically they behave invasive, show faster recurrence rates and

resistance to standard therapy options such as surgery and radiotherapy). [17, 30, 31]). The WHO defined aggressive pituitary adenoma by the combination of the following features: Rapid growth, radiological invasion, a high Ki-67 proliferation index. Adenomas with these features are labeled as “high-risk-adenoma” [10]. Therefore the evaluation of Ki-67 proliferation index is still recommended, but without a specific cut-off value [10].

Only 1% of all pituitary tumors are by definition pituitary *carcinoma*, which show neural or extra-neural metastasis (craniospinally or systemic) [10, 17, 30]. Pituitary carcinomas are usually functional, most often producing cortisol, prolactin and somatotropin in that order [32], citing [6, 24, 26]. However the definition of the WHO is independent of the histological subtype and no histological features can distinguish adenoma from carcinoma prior to metastasis [10].

As any other tumor pituitary tumors can be classified based on their responsiveness to classical treatment options. For instance, prolactinoma are regarded “refractory” if dopamine agonist therapy as well as surgical resection shows to be ineffective, as the tumor shows early recurrence and rapid regrowth even after repeated surgical resection/ radiotherapy [24, 27, 28]. It has to be noted though, that up to now, no consistent diagnostic/histopathological or clinical criteria have been defined for “refractory pituitary adenoma” [6, 24, 28]. Therefore, the true incidence of refractory PA is hard to be valued and the comparison in between the different clinical findings in studies regarding these tumors is difficult.

Table 1: Pituitary adenoma subtypes

Pituitary adenoma subtype	Prolactinoma	Somatotropinoma	Corticotropinoma	Thyreotropinoma	Non-functioning pituitary adenoma
Secreted hormone (independent of HPA)	Prolactin (PRL)	Somatotropin (syn. growth hormone)	ACTH	TSH	None (sometimes subunits of glycoprotein hormones [33]
Syndrome/ associated symptoms (exemplary)	Both sexes: Loss of libido infertility For women: Menstrual circle disturbances (secondary amenorrhoea/oligomenorrhoea) Galactorrhoea Men: Oligospermia Erectile dysfunction [34]	Acromegaly Change of facial features: - Enlargement of tongue, lip and nose - Prognathism - Prominent forehead Gigantism (if prior to closure of the epiphysis) Enlargement of hands and feet Diabetes mellitus Hypertension Sleep apnea Arthritis Carpal tunnel syndrome Cardiomyopathy (rare) Increased risk for colon neoplasia [22]	Cushing´s disease Change of facial features: - Plethora - Round face - Acne, hirsutism - Female balding Obesity/weight gain Dorsal fat pad Menstrual changes Hypertension Ecchymoses Striae Edema Lethargy, depression Abnormal glucose tolerance EKG abnormalities or atherosclerosis Proximal muscle weakness Osteopenia or fracture Recurrent infections Abdominal pain Headache, Backache [35]	General symptoms of Hyperthyroidism Weight loss Tachycardia Palpitations Tremor Hyperactive reflexes Nervousness/ depression fatigue Intolerance of heat Diarrhea [36]	Symptoms due to mass-effects: Headache Visual field defects (bitemporal hemianopsia) Ophthalmoplegia [22] Hyperprolactinemia (mild, due to stalk-effect) Hypogonadism Partial or complete hypopituitarism (with large NFPA)[33]
Treatment options	Dopamine-agonist therapy (first line, Cabergoline, Bromocriptine) Transsphenoidal surgery [34]	Transsphenoidal surgery (first-line) Cabergoline, somatostatin-analogues, pegvisomant	Transsphenoidal surgery (first line) Repeated surgery,	Transsphenoidal surgery (first line), after treatment of hyperthyroidism, reaching euthyroid state with	Transsphenoidal surgery Radiotherapy

		[22]	Bilateral adrenalectomy Ketoconazole Cabergoline [35]	methimazole, propyl-thiouracil; Somatostatin analogs, irradiation [22]	
Other specialities	See extra chapter on Prolactinoma	Patients with acromegaly have a 2-fold increase in mortality primarily to cardiovascular comorbidities. About two-thirds of somatotropinoma are macroadenoma [22]		TSH-producing pituitary adenoma are a rarity. The majority of them are macroadenoma and about 25% co-secrete growth-hormone or prolactin [22]	See extra chapter on NFPA

Table 2: Morphometric classification and the terms Invasiveness, Aggressiveness and Carcinoma in regard to pituitary tumors: The terms invasive and aggressive in regard to pituitary adenoma may not be used interchangeably. Currently, aggressiveness is primarily defined by its clinical behavior. Invasiveness is defined by radiological, surgical and histopathological findings. Pituitary carcinoma give rise to metastases, but often share histological features with aggressive adenoma (Table and content analog Di Leva et al., 2014 [7]).

Morphometric	■ Microadenoma (size <10 mm) ■ Macroadenoma (size ≥10 mm) ■ Giant adenoma (size >30–40 mm* or volume >10 cm^3)
Invasiveness	■ Invasion to surrounding structures (on the basis of radiological or histopathological investigations) ■ Invasion of the sphenoid sinus and/or cavernous sinus (25–55% of pituitary tumors) ■ Suprasellar extension of the tumor is not a criterion of invasiveness ■ In the Hardy classification system, invasive adenomas are grade III (focal bone erosion) and IV (extensive bone erosion of the skull base and parasellar structures) ■ In the Knosp classification system, grade 3 and grade 4 define true invasion of the tumor within the cavernous sinus ■ Macroadenomas can be invasive but are not often clinically aggressive
Aggressiveness	■ On the basis of clinical behavior* ■ Radiological signs of invasion ■ Rapid growth ■ Exhibition of high mitotic activity, ki-67 ≥3%, or p53 immunopositivity) ■ Non metastatic, but share common histological features with pituitary carcinoma *"Aggressive" adenoma are typically invasive giant- or macro- adenoma. Exceptions have been made, for certain types of microadenoma characterized by a high rate of recurrence or lack of response to therapy.
Carcinoma	Pituitary neoplasm wit neural or extraneural metastasis (craniospinal or systemic) independent of other histological findings.

1.2.3 Prolactinoma

Prolactinoma is the most common subtype of pituitary tumors, accounting for about 40% of all hormone-secreting pituitary adenoma [37]. Its main characteristic symptoms are caused by hyperprolactinemia. However, high PRL-levels can result from many physiological as well as pathological factors.

Hyperprolactinemia inhibits gonadotropin-releasing hormone pulsatile secretion and, consequently, FSH, LH, estrogen, progesterone and testosterone pulsatility thus leading to hypogonadism [38]. This results in fertility problems and sexual dysfunction for both sexes. For women, secondary amenorrhea or menstrual cycle disruptions lead to an earlier diagnosis as compared to men. In males the symptoms are less obvious than in women, still they can be the cause of infertility and impotence in about 11% of oligospermic men [39]. Erectile dysfunction and loss of libido are the main symptoms of hyperprolactinemic men [40]. Oligospermia and diminished ejaculate volume lead to fertility issues [38]. Less frequent symptoms include gynecomastia and reduced body

hair for men [40]. Galactorrhea has been reported in 30-80% of cases for women and 14-33%e for men [34, 41-43]. As hyperprolactinemia leads to sex steroid attenuation, bone loss occurs in both sexes not necessarily being restored after prolactin-level normalization [44].

The most common physiological causes of hyperprolactinemia in women are pregnancy and breastfeeding (Molitch et al. 2001, Biller et al. 1999, cited by [34]). Besides that, high stress- levels due to acute illness, exercise and psychological pressure can lead to hyperprolactinemia [34]. Surgery led to a significant elevation of serum-prolactin levels in both sexes, with higher rates observed for women in a study by Noel et al. in 1972 [45]. Same accounted for minimal-invasive procedures such as gastroscopy and exercise, to a lower degree [45]. Prolactin measurement is also often performed as an additional diagnostic tool for suspected epileptic seizure [46]. A retrospective study by Abuhuziefa et al. (2016) showed with class III evidence that postictal elevation of serum prolactin occurs in 84,4% of patients with epileptic seizures but also in 28,8% of patients with psychogenic non epileptic seizures [47]. Ictal activity spreading to the hippocampus or partial seizures involving the temporal lobes or limbic structures can elicit PRL release triggered by the hypothalamus via the HPA- axis [48].

The most common non physiological cause of hyperprolactinemia are side effects from drugs, mainly neuroleptics, antipsychotics and tricyclic antidepressants, dopamine-agonists, and with less frequency antihypertensive drugs such as verapamil ([34]. What is more, primary hypothyroidism, a very common endocrinological condition can lead to mild PRL increase [34].

After pregnancy, hypothyroidism and side effects have been ruled out, prolactinoma is the most likely cause of chronic hyperprolactinemia [34].

Estimated prevalence rates of prolactinoma range from 6-10 / 100 000 to 50 / 100 000 [44].

As pituitary adenoma in general, prolactinoma below 10mm diameter are called microadenoma [19]. Among prolactinoma, macroadenoma are more prevalent for men than for women. This is attributed to the delay of diagnosis because of unspecific symptoms as compared to women, but it might also reflect a gender specific difference in tumor pathogenesis [38].

Prolactin-levels induced by PA correlate strongly with PA diameter. Microprolactinoma usually result in PRL levels ranging in between 100-200 ng/ml. In 25% of cases PRL-levels below 100ng/ml are measured [34] Macroprolactinoma mostly show Prolactin-

levels over 250 ng/ml, reaching 20000 or more [34]. Especially for larger pituitary tumors with a size above 3cm the so-called hook effect when measuring PRL levels should be considered during the diagnostic procedure [34, 49]. Prolactin is commonly measured by clinical laboratories using two-site monoclonal sandwich assays such as immunoradiometric (IRMA) or chemiluminometric assay (ICMA). The hook effect is a known pitfall of immunometric assays [50]. They occur if antigen-levels such as Prolactin are extremely high outnumbering the capture and detection antibodies. As a result, Antigen binds separately to the respective antibodies, preventing the formation of a sandwich and thus detection [49]. That phenomenon has led to at least two reported cases of misdiagnosed macroprolactinoma as NFPA [49, 51]. The hook effect can be avoided by either using assays with two-step processing or by serial dilution with one-step assays [34, 49]. To unmask hook-effect with Prolactinoma, it is recommended to dilute serum-samples when measuring PRL [44], for instance with repeated measurement after a 1.100 dilution [34]. Especially for large or giant macroprolactinomas mild to moderate PRL-levels might otherwise be attributed to stalk effect and misdiagnosed as NFPA. Pituitary stalk compression due to intrasellar tumors like pituitary macroadenoma or craniopharyngioma can prevent tonic inhibition by dopamine via the pituitary stalk. It is a common cause for mild hyperprolactinemia in patients with NFPA [52]. Besides pituitary insufficiency macroadenoma might thus lead to lactotroph disinhibition and hyperprolactinemia. Hyperprolactinemia and related symptoms resolve after transsphenoidal surgery in most cases [53]. Still, because of laboratory pitfalls Prolactinoma might have be falsely classified as NFPA in the past before hook-effect has become known among physicians due to case-reports. What has to be taken into account as well is the presence of different circulating forms of PRL – while 85% of circulating PRL is normally monomeric (“little PRL” with a molecular mass of 23 kDa), serum also contains dimeric or polymeric forms of PRL, covalently bound to e.g. IgG, also called “big-PRL” (molecular mass 50-60 kDa) or “big-big-PRL” (150-170 kDa) [44, 54]. Macroprolactinemia is a common cause especially to asymptomatic hyperprolactinemia, though symptoms do occur in a smaller proportion of patients [44]. The lack of symptoms has been explained by the lower biological activity of macroprolactin [54]. According to retrospective studies the prevalence rate of macroprolactinemia is about 40% [44, 55, 56]. Screening for macroprolactin is possible with a polyethylenglycole (PEG) assay and has been recommended for

patients with asymptomatic hyperprolactinemia [44], some authors also suggest routine screening for macroprolactin in all patients with hyperprolactinemia [56].

1.2.4 Non- functioning pituitary adenoma

1.2.4.1 Definition and Overview

Clinically non-functioning pituitary adenoma are among the most common tumors of the sellar region [20]. As they do not secrete active hormones (at least on a clinically significant level) they are termed non-functioning. Thus, they do not present with clinically hypersecretory syndromes like functioning pituitary adenoma of the respective endocrine cellular origin do. They are either found incidentally on MRI performed for other reasons or diagnosed based on compressive symptoms such as headache, visual deficits or pituitary hormone deficiencies due to compression of the healthy pituitary gland [20].

Around three-quarters of these tumors produce intact gonadotropins or their glycoprotein subunits (alpha-/ or beta) as was shown by measurement of these products in the blood, by immunohistochemically staining for histological samples or by mRNA measurement in these tumors ([57], cites Young et al., 1996). Rarely, clinically NFPA stain positively for other pituitary hormones, such as STH, ACTH, PRL and TSH but do not secrete those hormones in clinically significant quantities. Thus they were called silent somatotroph, corticotroph, thyrotroph or lactotroph adenoma ([57], cites Yamada et al., 1991). A large percentage of around 30% had so far still be classified as “null-cell adenoma” [20] with negative hormone-immunohistochemistry results. However, new modern staining methods for lineage specific factors unmasked the cellular origin of the majority of those tumors. The majority of formerly called null-cell adenoma express SF-1 and therefore belong to the gonadotroph lineage. As more specific antibodies for lineage-specific transcription factors were developed and established in immunochemistry analysis, other silent hormone-secreting adenoma could be distinguished further diminishing the percentage of actual “null-cell-adenoma”. The WHO classification for pituitary tumors was revisited in 2017 accordingly [10-12, 20].

Essential diagnostics for NFPA include:

- MRI imaging of the sellar region to define tumor size and extension [20], also for the differential diagnosis other intrasellar tumors such as Craniopharyngeoma),
- Visual field examination

- Thorough assessment of the integrity of all pituitary hormone systems [20]

Transsphenoidal surgery is the first-line therapy for NFPA. However, complete resection can often not be completely accomplished and recurrence rate after surgery may be as high as 30% [20].
Recently it has become more and more evident, that many factors, such as cellular lineage and markers of cellular pathways can provide a better understanding of this heterogenous clinical subtype. By this, a better evaluation of prognosis as well as predictive markers of various second-line therapeutic strategies can be made. Also, new targeted therapies can hopefully be developed, improving patient outcome and survival. This includes the TGF signaling pathway, which has been shown to be involved in NFPA development by various authors. The evaluation of the TGF signaling pathway as a possible new treatment option for NFPA is the focus of this work. Before, NFPA will be more thoroughly illustrated.

1.2.4.2 Epidemiology

In a metaanalysis by Ezzat et al., PA were found in as many as 14,4% of postmortem samples and 22,5% of imagining studies, resulting in a total frequency of 16,7% [19]. According to an estimation of Katznelson et al. 1993, 25-30% of PA can be classified as clinically non-functioning as they do not lead to classical hypersecretory syndromes [33]. A large, community-based study in the UK on pituitary tumor prevalence and their subtypes supported this approximation, with 28% pituitary adenoma patients presenting with the non-functional tumor-subtype [58]. Nishioka et al. state that around 30-40% of surgically removed pituitary adenoma are clinically non-functioning as they lack clinical and biochemical evidence of adenohypophyseal hormone excess [10].

1.2.4.3 Characteristic signs and symptoms

Due to the lack of anterior pituitary hormone excess NFPA typically remain clinically silent tumors until mass effects occur, such as headache, visual deficits due to compression of the optic chiasm or cranial nerve palsies caused by invasion of the tumor into the cavernous sinuses [33]. This might be one of the reasons why non-functioning pituitary adenoma are the most frequently observed pituitary macroadenoma [23]. However a critical volume of the pituitary tumor is reached, compression of the adjacent pituitary gland often leads to partial or complete hypopituitarism [33]. When the pituitary stalk is compressed the dopamine

hypothalamic inhibitory function on prolactin-secretion is reduced, leading to mild hyperprolactinemia. This so-called “stalk-effect” has to be distinguished from falsely low PRL-measurements due to hook-effect with large Prolactinoma, as was mentioned above.

NFPAs are also diagnosed incidentally when a MRI is done for other signs or symptoms, therefore the number of NFPA in the general population might be higher [59]. According to Freda et al. 2011, only 10% of people with incidentalomas which are microadenomas experience tumor growth. As a result, experts recommend to observe the tumor size for progression at regular intervals before considering transsphenoidal surgery [22, 60].

However, a tremendous variability in tumor rates of growth must be noted, with tumor-doubling times ranging from 0.8- 27.7 years [22, 61].

1.2.4.4 NFPA subtypes and WHO classification

Morphologically and histologically, NFPA can be divided into distinct subtypes by various methods including immunohistochemistry, using antibodies against adenohypophyseal hormones, electron microscopy, in situ hybridization or reverse hemolytic plaque assay [10]. By measuring the expression-levels of transcription-factors characteristic for a specific cellular lineage of the anterior pituitary, NFPA could be divided into different cellular subgroups depending on their cellular differentiation profile and consequently their most probable cellular origin [10, 11]. Although some NFPA might not secrete hormones that can be measured by immunohistochemistry, they might still be a clinically silent differentiated counterparts of hormonally-active adenoma. By the complementary measurement of the distinct transcription-factors (PIT-1, SF-1, Tpit, ER α, also see table 3) characteristic of the respective cellular lineages many tumors that with regular IHC analysis have been classified as `null-cell-adenoma” could be unmasked as clinically silent pituitary adenoma (SPA) [10, 11] reducing the prevalence of the null-cell adenoma to below 1% approximately. Based on this, a new subclassification of NFPA was integrated into the WHO classification of 2017 on pituitary adenoma [10].

Table 3: IHC for PIT1 (pituitary-specific positive transcription factor 1; coded by the POU class 1 homeobox 1 gene), SF1, TPIT (coded by the T-box transcription factor 19 gene), and estrogen receptor- α (ER α) allowed for the identification of mutually exclusive lineage-specific markers in 95% of the cases, reducing the prevalence of the null cell adenoma subtype to ~1% [1, 2]

Cell lineage	Pituitary hormones by IHC	Transcription factors
Gonadotroph adenomas	FSHβ, LHβ, α- subunit	SF1, GATA2, ER α
Lactotroph adenomas	PRL	PIT1, ER α
Somatotroph adenomas	GH, α- subunit	PIT1
Thyreotroph adenomas	TSHβ, α- subunit	PIT-1, GATA2
Corticotroph adenomas	ACTH	tPIT
Null-cell adenomas	None	None
Pit-1-positive adenomas	GH, PRL, TSH β, α- subunit	PIT-1

Gonadotroph adenoma: Gonadotroph adenomas are the most common subtype of NFPA, making up around 75% of NFPA evaluated in a series of 516 NFPA-samples by Nishioka et al., 2015 [10, 11]. Diagnostic criteria for subclassification were immunoreactivity to gonadotropic hormones, pit-1 and SF-1. Most of the clinical characteristics of clinically non-functioning pituitary adenoma refer to that cellular subtype [10]. Though coming up as clinically NFPAs for lack of obvious endocrinological symptoms, gonadotrophin secretion, primarily FSH secretion, has been shown in a large proportion of those tumors both in vivo and ex vivo (Hanson et al., 2005, cited by [12]).

Before NFPA could be assigned to a specific cell-type by transcription factors it has already been documented in many cases that NFPA show α-Subunit production, common of glycoprotein-hormones, though no clinical evidence of hormonal hypersecretion could be determined [62].

Glycoprotein-hormones are heterodimers, consisting of one α- and one β-subunit. Every member of the large family of glycoprotein-hormones shares a common α-subunit and has a unique individual β-subunit [26]. There are only two cell types in the pituitary gland that produce glycoprotein-hormones: The gonadotropic cell-line (LH, FSH) and the thyreotropic cell line (TSH). Formerly the detection of the individual β-subunits of the respective glycoprotein-hormones in the serum was difficult [62]. As RIA became more advanced, the detection of the individual GP-hormonal subtypes became possible [63]. It could be shown that many (but not all) NFPA do in fact not only secrete α-subunits, but also free β- subunits or even intact FSH or LH [6, 24, 27,

63]. RNA-analysis and immunocytochemistry analysis showed similar results [5, 29-31, 63-65].

Because of that, GnRH-analogs, like nafarelin were tested for patients with NFPA in clinical studies [66]. For instance, in the study- group of Colombo et al. patients not only suffered from NFPA but also showed hypogonadism. Though α-SU levels increased, the overall LH and FSH levels decreased in most patients [66]. These findings were confirmed by other authors.

α-subunit expression does appear in ontogeny before β-subunit expression [66, 67]. It was therefore assumed that the potential adenoma cell is a gonadotroph cell being arrested in an early developmental stage [68]. Normal gonadotroph cells secrete functioning heterodimers of LH and FSH which are bioactive on the level of gonads. In contrast, non-functioning gonadotropinomas only secrete the free gonadotropin- alpha and beta-subunits, which are biologically inactive at the gonadal level [33]. This model is illustrated in figure 2.

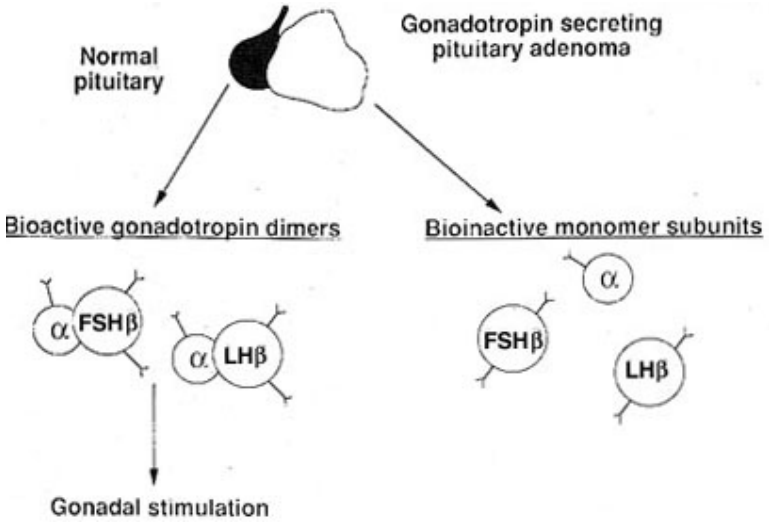


Figure 2: Secretion of gonadotroph free subunits by clinically non-functioning gonadotropinoma, the most common subtype of NFPA (Figure taken from Katznelson et al.). Physiological, gonadotroph pituitary cells secrete intact LH and FSH heterodimers which are bioactive at the gonadal level. In contrast, clinically nonfunctioning pituitary adenomas secrete just the free gonadotropin alpha- and beta-subunits, which are bioinactive at the gonadal level [6].

Silent corticotroph adenoma (SCA): In most cases, corticotroph adenoma can be distinguished from other NFPA by ACTH immunoreactivity [10]. However, Nishioka et al. could show that around 25% of hormone-negative adenoma expressed Tpit and showed increased POMC-mRNA expression, both being markers of corticotroph cells [11]. Silent corticotroph adenoma (SCA) with ACTH immunoreactivity of histological samples are well-known for their aggressive behavior without any clinical signs of

Cushing´s disease and no elevated blood levels of ACTH and Cortisol thus making diagnosis difficult [69]. With the measurement of Tpit, an even higher percentage of SCA could be found in between clinically non-functioning pituitary adenoma. These tumors are often large, lobulated and mostly affect patients younger than 40 years with a female preponderance [10, 70]. The new 2017 WHO classification graded SCAs as "high -risk adenoma" due to their aggressive behavior and high recurrence rates [71] as was suggested by works from some authors [10, 11]. However, a large metaanalysis by Fountas et al. 2018 showed no evidence supporting higher recurrence risk of SCAs compared to other NFPA subtypes [71].

Silent pituitary adenoma of pit-1 derivation: This complex subtype makes up around 9% of clinically non-functioning pituitary adenoma. They most probably derive from a mammosomatotroph stem cell with pit-1 being a marker of GH/PRL/TSH lineage [10]. These tumors show a heterogeneous morphology as they include plurihormonal pit-1 positive adenoma, silent somatotroph, lactotroph and thyrotroph tumors. As with SCAs, silent pit-1 adenoma mostly affect younger patients, are typically large, aggressive tumors with high recurrence rates after surgery [10, 11, 70].

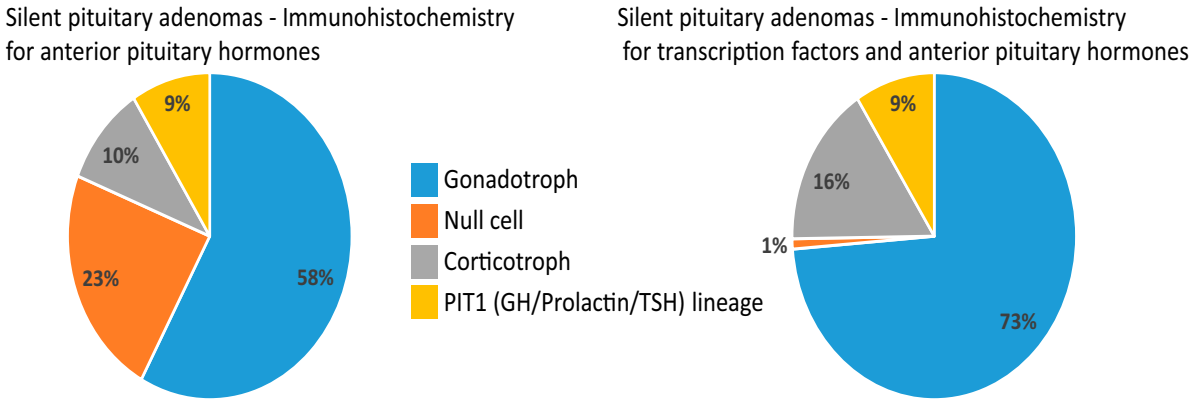


Figure 3: Schematic illustration of the percentage of clinically non-functioning pituitary adenoma identified by solely immunohistochemistry for anterior pituitary hormones alone (left) and with the inclusion of transcription factors for diagnosis (right) (Drummond et al., 2018 [2]).

Null-cell adenoma: In 2004, the WHO classified null cell adenoma as "hormone-immunonegative adenoma, with scattered cells that are immunopositive for glycoprotein hormones" (Lloyd et al., 2004, cited by [20]). However, as has more recently been illustrated most of those tumors produce SF-1, a transcription factor specific for the gonadotroph lineage [11, 20]. As the measurement of specific transcription factors using antibodies has become more established the estimated prevalence rate of null-cell-adenoma reduced to a level below 1% [12]. As a result, the

concept of “null-cell-adenoma” was revisited by the WHO classification 2017, defining it as a tumor without IHC evidence of cell type differentiation and also negativity for lineage-specific transcription factors (Lloyd et al., 2017, cited by [16]). A schematic illustration of the percentage of the individual non-hormone producing tumor entities is shown in figure 3.

1.2.4.5 Invasive and aggressive pituitary adenoma

About 35% of pituitary adenoma are invasive [72]. In clinical practice, classification systems like Hardy´s classification, Wilson-Hardy-classification and Knosp classification are used to describe the extent of the invasion. Due to the infiltration of the surrounding sensitive structures surgical removal of invasive pituitary adenoma is challenging but essential [72]. The distinction in between “invasive” and “aggressive” is based solely on the lack of metastasis for invasive pituitary adenoma. For the current definition of invasive/aggressive PA by the WHO also see table 2. Despite the differences in clinical behavior, invasive and aggressive pituitary adenoma do most likely share the same molecular basis to a certain extent [72]. Distinct molecules like “hypoxia-inducible factor-1α, pituitary tumor transforming gene, VEGF, fibroblast growth factor-2, and matrix metalloproteinases (MMPs, mainly MMP-2, and MMP-9) have already been identified to be important contributers to the invasiveness of pituitary adenomas [72].

TGFβRII is less expressed in invasive non-functioning pituitary adenomas [72].

1.2.5 Conventional treatment options for pituitary tumors

1.2.5.1 Prolactinoma therapeutic guidelines

For symptomatic Prolactin secreting micro- and macroadenoma, dopamine agonist therapy is the first line of therapy recommended by the US- Endocrine Society, in order to lower prolactin levels and decrease tumor size [44]. Cabergoline is recommended before Bromocriptine, as it shows to be more effective [44]. This is attributed to a higher affinity of cabergoline to dopamine-receptor binding sites. Besides, Cabergoline shows lower rates of side effects as compared to bromocriptine [44]. Transsphenoidal surgery is to be performed only if prolactin levels do not drop even with escalated doses of dopamine agonists, no significant reduction in tumor size is observed or if intolerable side effects of dopamine agonists do occur [44]. It has to be considered that protracted use of high dose- cabergoline poses the risk of valvular regurgitation [44]. Standard

doses of cabergoline for prolactinoma treatment do not heighten the risk of valvular disease as multiple studies suggest [44].

1.2.5.2 Dopamine agonist resistant Prolactinoma

Prolactinoma that fail to respond to *dopamine agonists are called dopamine agonist resistant prolactinoma* (DARPs). Dopamine agonist resistance is defined by failure to normalize PRL levels and failure to decrease tumor size by ≥50% [41]. This is seen in 11% of prolactinoma patients treated with cabergoline and to an even higher percentage for bromocriptine and pergolide [41]. One-third of those treated with bromocriptine and 10–15% of those treated with pergolide or cabergoline show a limited decrease of tumor size below 50%. Study results suggest that different mechanisms underlie dopamine resistance in prolactinoma [44].

Studies of *in vitro* cell preparations show that the D_2 receptors of resistant tumors are decreased in number but have normal affinity [44].

Dopamine inhibited adenylate cyclase activity by 28.8 +/- 5.6% in five bromocriptine-responsive tumors and by 16.5 +/- 4.3% in adenomas from eight resistant patients. In contrast, in the five patients whose tumors grew during therapy dopamine paradoxically stimulated adenylate cyclase activity (+26.4 +/- 9.8%) [73].

t resistance to bromocriptine therapy results from deficient dopaminergic regulatory mechanisms of adenomatous cells. The major abnormality seems to be a decrease in D2 dopamine receptors, but the phenomenon also may be associated with a postreceptor defect [73].

For young women at child bearing age who want to conceive, clomiphene, gonadotropins and GnRH are alternative treatment options. For women who don´t want to conceive hormonal contraceptives or estrogen replacement may be used [41, 44]. For macroadenoma though, which show a continuous growth or already show symptoms due to mass effects transsphenoidal surgery is necessary if dopamine-agonists show no effect [41].

1.2.5.3 Treatment of hormone-secreting adenoma other than prolactinoma

Minimal-invasive transsphenoidal resection is the first line therapy for other types of benign FPA than Prolactinoma. Unfortunately, tumors with more aggressive/invasive behavior often show high recurrence rates even after repeated surgeries. Radiotherapy is sometimes used against PA with aggressive/ invasive behavior. However, prolactinoma are usually not radiosensitive ([6, 21, 28], Molitch 2014, cited

by [21]). Temzolomide, which is the standard- therapeutic agent against glioblastoma, has been used for the past decades to treat aggressive/ refractory PA [74] with limited success ([21]. Still, NFPA do not show good response to TMZ as compared to FPA [74]. Due to this scientists and doctors are still looking for alternative treatment options especially for NFPA.

1.2.5.4 Treatment of NFPA

So far, treatment options for clinically NFPA include:

- No treatment with careful observation
- Surgery, most commonly via transsphenoidal resection (with/without radiotherapy postoperatively)
- Radiotherapy alone
- Medical therapy [57]

For incidentally found lesions on MRI scans surgery is restricted to cases where mass-effects can be unmasked by testing for hypopituitarism and visual deficits by the ophthalmologist [22, 60]. Only around 10% of incidentally found NFPA on MRI or CT scans done for other reasons show significant tumor growth. Therefore, repeat MRI controls and thorough assessment of hormonal state is recommended before surgical treatment (also see flow chart on Fig. 4) [22]. If a NFPA becomes symptomatic due to mass-effects or shows enlargement transsphenoidal surgery is usually recommended as first-line therapy. Clinical apparent NFPA are the most frequent macroadenoma. Therefore, surgical resection without any tumor remnants is challenging. NFMA regrow in 12-58% of cases during 5-year follow-up [23, 75-77]. If hormonal deficits manifest due to compression of the pituitary stalk resolution of 1 or more hormonal deficits after surgery are to be expected in 15-50% of cases [22, 57]. Hyperprolactinemia due to stalk-effect dissolves in about 75% of patients after surgery [22, 57]. However, in 2-15% of patients the opposite occurs with an additional hormone loss in 2-15% of patients postoperatively as compared to preoperative hormonal state [22, 57]. Radiotherapy alone is rarely done and mainly restricted to patients with contraindications against surgical treatment [22]. Radiotherapy is mainly done as an adjunctive treatment, especially if r0 resection is not possible and residual tumor mass remains visible on postoperative MRI scans [22]. However, long-term or repeated radiation therapy of the brain and pituitary stalk carries the risk of various

complications, such as hypopituitarism, neurocognitive dysfunction, cerebrovascular disease and long-term risk of secondary intracranial neoplasms [23]. Brochier et al. could reveal some predictors for recurrence of NFMA after surgery in a series of 142 patients with a 0.5-30 years follow-up. This included cavernous sinus invasion, no immediate postoperative radiotherapy, postsurgical remnant and positive immunostaining for hormones other than gonadotropins [23]. Medical therapeutic strategies with dopamine- D2-recpeptor agonists have been tried [22], showing reduction in tumor volume in some series [78, 79]. However, in an in vitro study with 20 NFPA -samples by Gagliano et al., 2012, only 55% were positive for DR2. Only for those samples, Cabergoline a significant effect om cell viability and a 20% reduction of VEGF-secretion was observed [80]. Despite the small sample-collective, this study suggests that Cabergoline will only be effective in a selective number of NFPA. In another study, Somatostatin (SRF) and the Somatostatin-analog pasireotide were tested against NFPA in vitro. The antiproliferative effect and VEGF-inhibition also worked only in a “responder-group”, which lacked a certain Somatostatin-receptor subtype, SSTR5 [81]. In both studies, the inhibition of VEGF-secretory function correlated with the antiproliferative effects of the respective agents [80, 81].

Still, long-term follow-up treatment options, for patients after surgery or for patients with contraindications against surgery, non-invasive treatment options are still lacking.

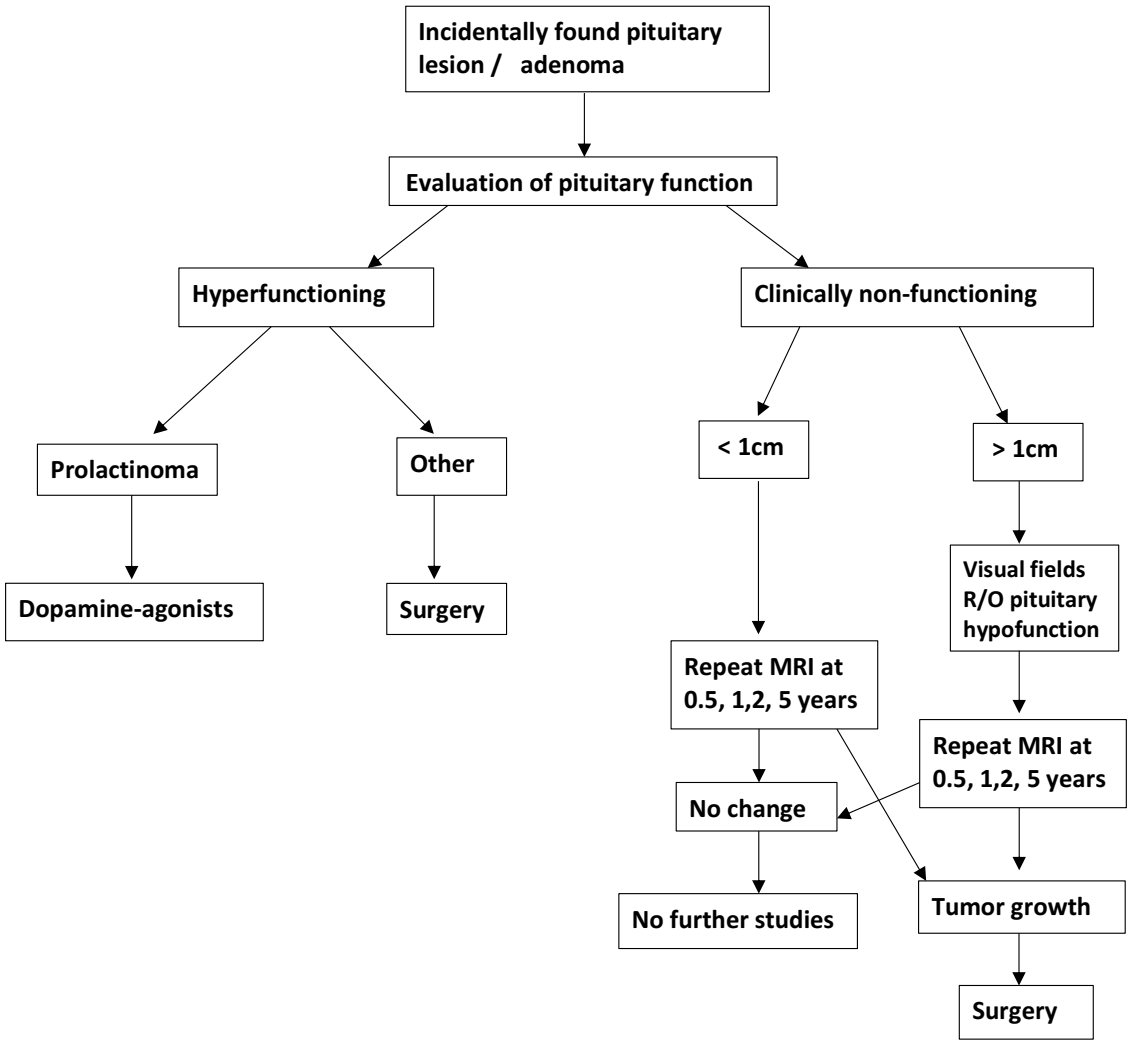


Figure 4: Systematic flowchart of clinical procedure guidelines for incidentally found pituitary lesions [8, 9]

1.2.5.5 Treatment of aggressive pituitary adenoma

For clinically aggressive as well as invasive pituitary adenoma and carcinoma, surgery is the first line of treatment to be performed, typically in combination with radiotherapy. If the tumor reoccurs frequently even after secondary surgery, alternate treatment regimens are considered. Some aggressive PA show response to Temozolomide, a second-generation alkylating agent commonly used in chemotherapy for glioblastomas. Treatment with Temozolomide is also sometimes recommended for DARPs and clinically non-functioning pituitary adenoma. The timing for initiating treatment, its duration and consequences is still under debate and Temozolomide is still regarded as a last option [82].

Certain variables about problematically behaving pituitary tumors, such as large NFPA, invasive and aggressive pituitary adenoma and carcinoma are still unsolved.

Therefore, the development of new targeted therapies has been the focus of researchers for the past decade. Many new targeted therapies have been proposed, such as mTOR-Inhibitors (everolimus), monoclonal antibodies against VEGF (bevacicumab) and EGFR inhibitors [82]. Still, no targeted therapeutic strategy has become clinically established so far.

1.3 The TGFβ signaling pathway

The TGFβ signaling pathway is a commonly used essential cellular pathway, known to play a role in various organs, tumors, immune-modulating pathways and embryogenesis [26]. TGFβ is a cytokine binding to its corresponding receptor subtypes. There are more than 30 molecules known to belong to the TGFβ superfamily [26] [83].

1.3.1 TGFβ superfamily, structure and secretion pattern

The TGFβ superfamily consists of structurally related cytokines with 66-60% sequence identity and nine strictly conserved cysteines [84], also belonging to the larger cystine-knot-growth factor family [85]. They are dimers of protein subunits (homo- or heterodimers) [85], which are linked by a single disulfide bond provided by a cysteine residue [84]. The other 8 evolutionary conserved cysteines within the respective subunits form intrachain disulfide bonds, leading to a characteristic cysteine-knot structure [84]. Only by linkage of the two subunits with the 9th cysteine disulfide bridge, the dimeric cytokine becomes functionally active, though. An overview of the members of the TGFβ- superfamily is shown in table 4.

Activin and Inhibin mainly use Smad2 and 3 as downstream mediators, with Smad7 acting as their inhibitory-Smad. BMP, on the other hand, relies on Smad1, 5 and 8 as their downstream mediators and the inhibitory function of Smad6.

Table 4: Overview of the TGFβ-superfamily

Cytokine	Structure (exemplary)	Receptor (exemplary)	Downstream (Smad-) mediators	Expressed in (exemplary)	Role/ function (exemplary)
Activin	Activin A β_A β_A Activin AB β_A β_B Activin B β_B β_B [86]	ActRIIA, ActRIIB + ActRIB, ALK4 (activin-receptor-like kinase 4), ActRIC (ALK7) [87-90]	Smad2 Smad3 Smad4 [91] Smad 2/3 lead to a higher expression of Smad 7 (inhibitory Smad) as a negative feedback-mechanism [86, 91, 92].	Gonads, placenta, pituitary gland, skin, dendritic cells [86].	Stimulates FSH secretion in the gonads; Spermatogenesis, enhancement of androgen production (vial LH/FSH), enhancement of FSH binding in the ovarian follicle, wound healing (skin), activation of fibroblasts [86].
Inhibin	Inhibin A α β_A Inhibin B α β_B [86]	Betaglycan (TGFβRIII) InhBP/p120 ActRIIA ActRIIB [89]	Mostly antagonistic role of Activin and BMP, e.g. binding activin receptors with low affinity without activation of downstream mediatiors by phosphorylation, therefore inhibition of activin signaling.[86, 89], though evidence for independent signaling via an independent receptor does exist [93].	Gonads, placenta, pituitary gland, skin, dendritic cells [86, 89, 93]	Regulation of gonadotropin, Sertolli cell function, Leydig-cell function [89]follicle development [86, 94-96]
BMP (Bone morphogenic protein)		BMPR (bone morphogenic protein receptor)1-7, 8a/b, 10,11,15 [97]	Smad1 Smad5 Smad8 [91, 98], Smad6 (inhibitory Smad)[91].	Embryonic cells, ovarian follicles, bone and cartilage tissue, Hippocampus [99-102]	Embryonic development, bone and cartilage formation and healing [99-102]
AMH (Anti-Müllerian-Hormone)		Serine-threonine kinase receptors type I and type II, the type II receptor imparts ligand	BMP-specific R-Smads and reporter genes [104]	Gonads [105].	Inhibition of proliferation of Leydig-cells and testosterone production, prevention of the recruitment

		binding and the type I receptor mediates downstream signaling when activated by the type II receptor [103]			of primordial follicles [105]
GDFs (growth-differentiation factors)	Isoforms 1-9	TGFβRI (for GDF-9), BMPRII [106]	Smad3 (for GDF-9) [107], Smad1,5,8 [108, 109]	Synovial cartilage [110], ovary [111], central nervous system [112].	Angiogenesis, skeletal development, inflammation, cancer, folliculo-genesis [111, 113, 114]
GDNF (glial derived neurotrophic factors)	E.g. GDNF isoforms 1 and 2	Glycosyl-phosphatidylinositol-(GPI) anchored receptors, termed GDNF-receptor α-1 in collaboration with signaling receptor subunits, such as Ret-tyrosine-kinase [115].	Smad2 Smad3 [116]	Glial development [115], liver fibrosis [116]	Putamen [115], liver (hepatic-stellate cell) [116]
TGFβ (transforming-growth factor)	TGFβ-1, -2, -3	TGFβRI, TGFβRII, TGFβRIII (betaglycan)	Smad2 Smad3 [91]	Pituitary gland [117, 118], kidney [119], midbrain [120], regulatory T-lymphocytes [121]	Kidney injury/disease and renal inflammation [119], regulation of immune responses [121], dendritic growth and survival of dopaminergic neurons [120]

There are 3 isoforms of TGFβ (TGFβ-1, -2, -3). Their individual precursor molecules contain a propeptide-sequence which is referred to as latency associated peptide or LAP. LAP is proteolytically split from the mature TGF signaling protein, but remains associated to it via noncovalent interactions. In the endoplasmatic reticulum (ER). LAP is bound to latency binding protein (LTBP) [26]. LTBP regulates the secretion, storage and activation of the TGFβ-LAP complex [26].

Several endogenous activators of the TGFβ- release have been identified [26]. However, their meaning and function in the context of the individual tissues are not fully understood [26]. Once released from the ECM the respective TGFβ- subtypes bind to their corresponding receptors.

1.3.2. TGFβ receptors

As was already mentioned, members of the TGFβ-superfamily signal via binding to dual specificity kinase receptors at the surface of the target cells [90]. With only 12 members, the family of serin-threonin-kinases in mammals is rather small, as compared to tyrosine-kinase-receptors with 58 members (Heldin et al., 2014, cited by [90]). Of those 12, 7 are type-I and 5 type-II receptors [90]. When a cytokine of the TGFβ-superfamily binds to a receptor, a heterotetrameric complex of two type I and two type II receptors is formed [90]. The two type-II-receptors phosphorylate and thus activate the type-I-receptors (also see figure 5). Then, the activated type-I-receptors induce phosphorylation of the pathway restricted Smads and give way to the intracellular part of the signaling pathway [90]. TGFβRIII is a membrane-glycoprotein which facilitates TGFβ binding to the type-II receptor [21].

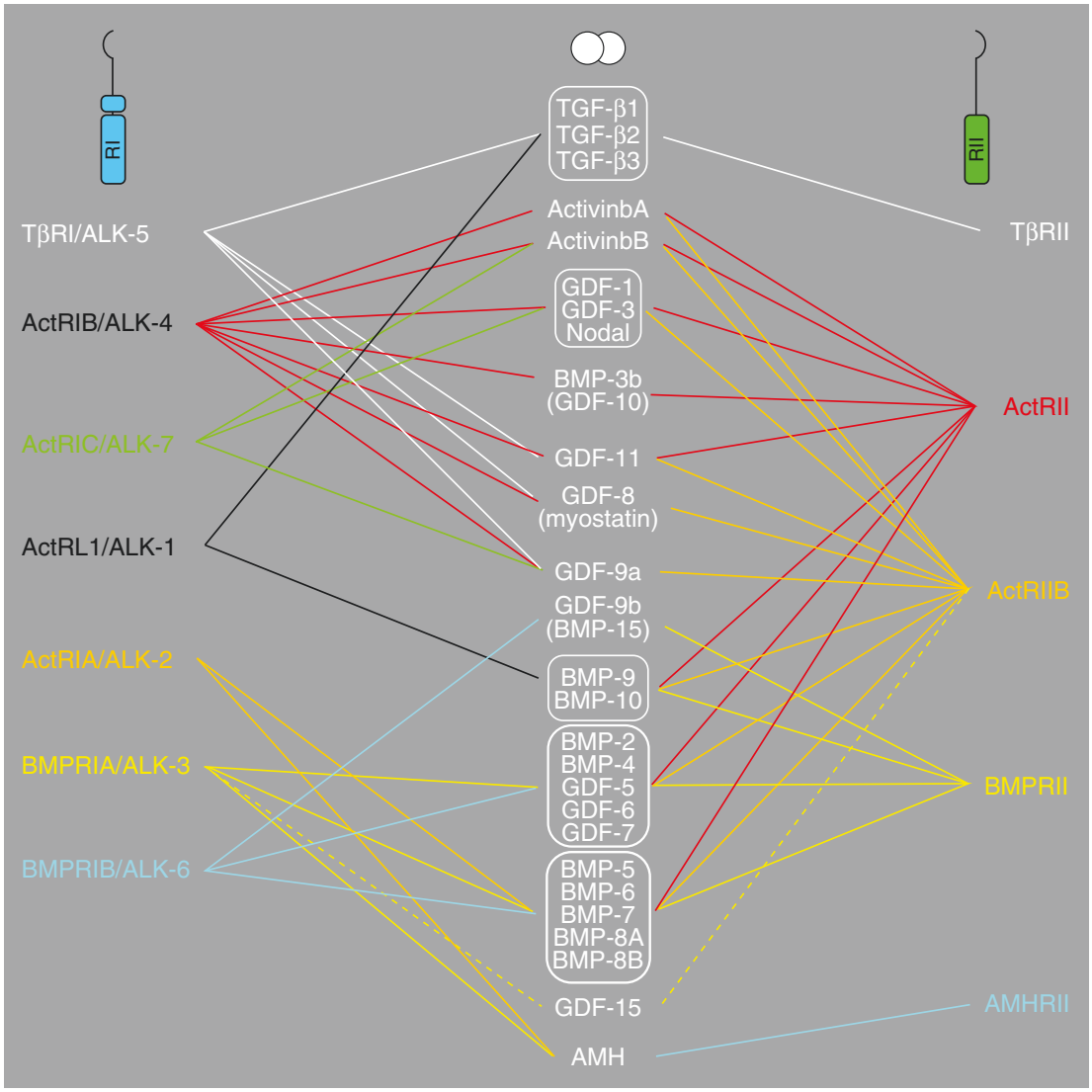


Figure 5: Schematic illustration of the type-I and type-II-receptors and the selective binding of the respective members of the TGFβ-superfamily [90]

First, TGFβ mainly binds to the type II receptor. Triggered by this the TGFβRII phosphorylates and forms a complex with TGFβRI [5, 122]. Thus, the intracellular part of the TGFβ signaling pathway takes its course. As far as we know, for the three TGFβ-isoforms, TGFβ-1, -2, and-3, there are two type-I, but only one type II receptor (TGFβRII). The most common type-I receptor is TGFβRI, or ALK-5 (activin-receptor-like-kinase). The alternate type-I-receptor is ALK-1, which is expressed e.g. in endothelial cells [90]). TGFβ-1 and TGFβ-3 bind TGFβRII with higher affinity than TGFβRI, as compared to TGFβ-2 [90]. Therefore, TGFβ-2 needs either preformed TGFBRII-TFRI-complexes or the help of TGF-coreceptors [90]. There are various coreceptors or modulators of TGFβ-signaling. One of those coreceptors is betaglykan, also named TGFβRIII [90]. Depending on expression levels and subcellular localization, betaglycan can promote or suppress TGFβ- signaling [90]. For instance,

overexpression of betaglycan in MDA-MB-231 cells inhibited TGFβ-induced Smad2/3 phosphorylation in an experiment by Tazar et al., 2015 (Tazar et al., 2015, cited by [90]). Loss of basolateral localization in polarized breast epithelial cells promoted EMT [90]. Betaglycan can bind all three TGFβ -isoforms, stabilizing the complex, which is of particular importance for TGFβ-2 because of its low intrinsic receptor affinity [90]. As Stenvers et al. could show, betaglycan-deficient mouse embryofibroblasts show reduced Smad2 translocation in the nucleus, leading to reduced growth suppression in response to TGFβ-2 stimulation, but not in response to TGFβ-1 and TGFβ-3 [90].
As was already mentioned, the activated type I receptor is primarily responsible for the activation of the Smad-proteins. This happens by phosphorylation of the Smad-proteins on two Serines on the SSXS motif at the C-Terminus of the respective Smads. The activated Smads (R-Smads) form a complex with Smad4. Unlike the other Smad-proteins Smad4 can be understood as an essential mediator of the cascade, thus nearly always being involved in the distinct Smad-induced pathways. By binding to Smad4 the respective Smads are enabled to go to the nucleus [122]. There, they act as specific transcription factors, either interacting with other specific DNA-binding transcription factors such as *Mixer* [122] or more directly, by binding to DNA (e.g. *CAGA Smad-binding element* specific for Smad3/4 – complexes) [122].

1.3.3 TGFβ-induced Smad signaling and alternate intracellular pathways

In short terms, Smad proteins act as shuttle proteins of the TGFβ signaling pathway. The term “Smad” is a. merger of two names for the gene of two orthologous proteins: Sma from *Caenorhabditis elegans* and Mad from *Drosophila melangolaster* [123, 124]. The Smad-family consists of 8 members, which have to be subdivided by their intracellular role into three different classes [124]:

- Pathway restricted or receptor regulated Smads (R-Smads) -> Smad 1 Smad2, Smad3 and Smad5 that directly interact with the activated TGFβIR
- Common mediator Smad (C-Smad or Co-Smad) -> Smad4 acting as the crossing link/mediator of the pathway
- Inhibitory-Smads (I-Smads) -> Smad6, Smad7 that inhibit TGFβ, Activin and BMP-signaling by binding to the type IR and interfering with the phosphorylation of the pathway-restricted Smads [125]

TGFβ, Activin and Inhibin mainly use Smad2 and 3 as downstream mediators, with Smad7 acting as their inhibitory-Smad. BMP, on the other hand, relies on Smad1, 5

and 8 as their downstream mediators and the inhibitory function of Smad6 [91] (also see table 4).

The pathway-restricted Smads, in case of TGFβ mainly Smad2 and Smad3, are phosphorylated by the receptor at a C-terminal SSxSmotif. This relieves a self-inhibitory-function and promotes the association with the common Smad, Smad4 [91]. Smad4 is used by all members of the TGFβ-superfamily as a shuttle protein in between the nucleus and cytoplasm. Smad4 forms heteromeric complexes with other activated (phosphorylated) Smads. This complex translocates into the nucleus and, from there, regulates the transcription of target genes [124] in conjunction with other transcription factors and with the recruitment of other co-activators and co-repressors [91]. Smad4 can freely transfer nucleus/cytoplasm, independent of the TGFβ signaling pathway [124, 126]. In unstimulated cells it is, predominantly localized in the cytoplasm. This is attributed to a unique leucine-rich nuclear export signal. While the nuclear import of Smad4 happens spontaneously, CRM-1 (*chromosome region maintenance 1*) exports Smad4 out of the nucleus [126]. This export is suppressed in the presence of TGFβ/ activated state of the signaling pathway [126]. Inman et al. assumed that Smad4 complexed with phosphorylated Smad2/3 loses its CRM-1 binding site thus leading to nuclear accumulation, at least until the complex dissolves [126, 127]. The dephosphorylation and nuclear export of Smad2 and Smad3 is independent of Smad4 and CRM-1 [126].

The gene for Smad4 or formerly often called DPC4 (deleted in pancreatic carcinoma locus 4) is a well-known tumor suppressor gene, best recognized in pancreatic cancer, but also in juvenile polyposis syndrome, prostate and colorectal cancer [124].

The activation of the Smad2/3 pathway by TGFβ parallelly induces the expression of the inhibitory Smad7, as a downstream feedback inhibitor [91]. Smad7 transiently associates with phosphorylated type I receptors (ALK4 or ALK5), interfering with further Smad2/3 activation [91]. Another mechanism by which Smad7 inhibits further signaling is through binding and targeting Smurf1 to the plasma membrane, thus facilitating type I receptor ubiquitination and internalization [91]. Besides Smad7, TGFβ-signaling is further controlled by dephosphorylation and ubiquitination of the respective P-Smads [91]. Smad7 interacts with GADD43 (*growth arrest and DNA damage protein*), a regulatory subunit of the protein phosphatase 1 (PP1) holoenzyme, which recruits PP1c (protein phosphatase 1c). The Smad7-GADD43-PP1c complex then dephosphorylates TGFβRI, thus inactivating it [128]. An important modulator of

the inhibitory function of Smad7 is the protein SARA, which is further discussed below [129].

Recent research revealed a number of additional factors interacting with the Smads that can regulate the signaling outcome [129]. One essential modulator if not co-receptor is betaglycan, which was already discussed above. Another important modulator of the signaling-pathway is SARA (*Smad-anchor for receptor activation*), a protein with four essential functional domains [129].

- FYVE motif
- Smad binding domain (SBD)
- PP1c-binding domain (PBD)
- C-terminal

The C-terminal region of SARA directly interacts with the TGFR [129]. With its SBD-domain, SARA directly interacts with Smad2 and Smad3 in their unphosphorylated state. Once phosphorylated, Smad2/3 dissociate from SARA to form a complex with Smad4 [129]. Through SBD, SARA leads to balanced distribution of Smad2/3 in the cytoplasma and presents them to the TGFβ-receptor for activation [129]. SARA is also thought to play an essential role in Smad2-activation, for instance by masking its nuclear import signal. Although SARA is not essential for Smad3/ TGFβ-mediated signaling as compared to Smad2, but it indirectly affects Smad3-signaling as well by modulating the balance in between Smad2 and Smad3 [129]. Via PBD SARA recruits the catalytic subunit of protein phosphatase 1 (PP1c) thus playing an important role in the dephosphorylation of TGFβRI mediated by Smad7 [129]- SARA most likely serves as an anchor protein, enhancing the availability of PP1c to GADD34 [129].

The FYVE motif is a zinc-finger-like structure that binds Phosphatidyl-inositol-3-phosphate, which then directs the holoenzyme SARA to early endosomal compartments. From this localization, it can directly interact with both, TGFβ-receptors and Smad-proteins [129].

Various other modulators or interactors of the intracellular part of the signaling pathway have been identified, including, for instance, Daxx, Hgs/Hrs (hepatic growth factor-regulated tyrosine kinase substrate), disabled-2 (Dab2), TGFβ-receptor-I associated protein-1 (TRAP-1), and TRAP-1-like protein (TLP) [130].

It has also to be noted that TGFβ is involved and can directly activate Smad-independent cellular pathways, such as MAPK, PI3K, Akt/PKB, NFκB,Wnt/β-catenin

pathways [21]. The TGFβ-superfamily can e.g. induce the activation of all three known MAP-Kinase pathways [122]. Evidence suggests that the respective signaling pathways are interlinked on a subcellular level. For instance, the activation of the JNK and p38 MAPK through TAK1 (TGFβ activated kinase 1) by TGFβ-receptors is regulated by the inhibitory Smad6 and Smad7 [131]. TGFβ-induced p38 MAPK and JNK-signaling also seems to play an important role in the regulation of epithelial-mesenchymal-transition (EMT), an important step towards cancer cell invasion and metastasis [131], although this process is regulated by Smad-dependent signaling as well [132]. These additional alternate TGFβ-induced signaling pathways as well as their interlinks have to be taken into account as well.

For an overview of Smad signaling and other signaling pathways triggered by TGFβ see Fig.6

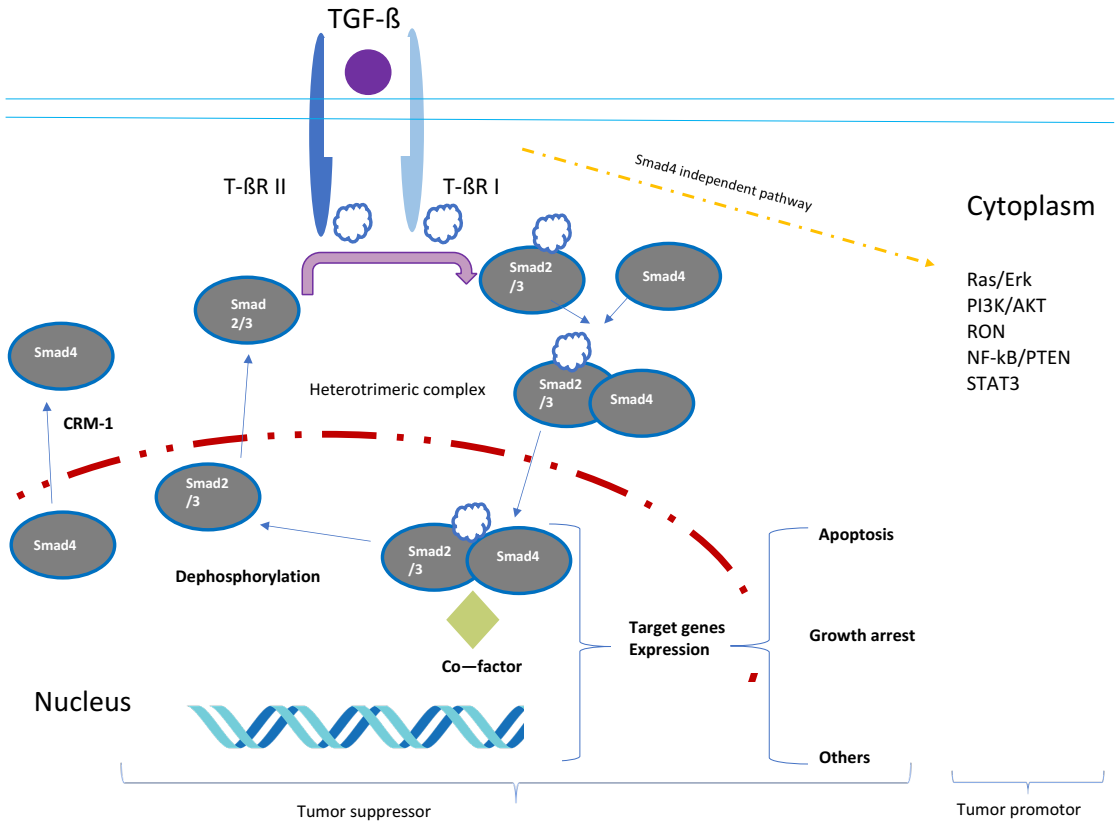


Figure 6: Schematic illustration of the Smad dependent and Smad independent TGFβ induced intracellular pathways adapted from Xia et al., 2015 [4].

1.3.4 The TGFβ signaling pathway and tumorigenesis

TGFβ is known to play a dual role in tumorigenesis – it can both act as a promotor and suppressor on tumor development [5], as has been confirmed by many authors [5, 67,

133-135]). Increasing evidence suggests that it does not only depend on the respective tumor-type but also on the stage of tumorigenesis [5, 30, 65].

In non-malignant epithelial cell lines TGFß has been considered a tumor suppressor based on its anti-proliferative effects [136]. It plays a role in maintaining tissue architecture and inducing apoptosis [137] thus inhibiting tumor development [137]. By distinct alterations within this pathway, however TGFß loses its anti-proliferative effect and even becoming a tumor promoter instead of tumor suppressor, e.g. by fostering angiogenesis, invasion and suppressing immunosurveillance [137]. The TGFβ-signaling is known to play an essential role in epithelial-mesenchymal transition (EMT) which is an important step towards metastasis, in which Smad4 represents the key component [138]. As was already mentioned, TGFβ is also thought to play a key-role in the development of cancer-associated-fibroblasts (CAFs) [139]. To reverse the tumor-promoting effects of the TGFß signaling pathway while retaining its protective effects against tumor formation will prove to be challenging. This is complicated by the fact that the individual alterations in the TGFβ signaling pathway depend largely upon the respective tumor-types. Distinct up- and downregulations of TGFβR and Smad/ p-Smad proteins have been described [58]. Thus, examining and understanding the role of the TGFβ signaling pathway in the respective tumors types is tricky in general. This accounts for the pituitary gland as well.

One common feature of the majority of cancers seems to be the relative insensitivity towards growth inhibition by external application of TGFß [140] which is attributed to loss-of-function mutations in TGFβRII, Smad2 and Smad4 genes [140]. On the other hand, elevations of TGFβ levels have also been associated with cancer initiation and progression [140]. Inhibiting agents of the TGFß signaling pathway have led to a reduction of tumor size, progression and aggressiveness in various tumors, e.g. pancreatic and breast cancer, targeting distinct extra- and intracellular components of the pathway [140]. In order to specifically target the tumor-promoting components involved in the respective tumors small-molecule inhibitors will be the most suitable in providing possible new treatment options as tumor-suppressive effects of the pathway should not be altered. SB431542, an inhibitor of TGFβRI-kinase activity has been shown to suppress tumor-promoting effects of TGFß such as cell motility, migration, invasion and VEGF-secretion in various human cancer cell lines [137]. SB-431542 could e.g. completely block TGFß-induced migration of lung adenocarcinoma cell lines [137]. On the other hand, SB-431542 can also block TGFß-induced apoptosis and

antiproliferative effects [137]. Whether SB-431542 can act as a tumor-suppressor or inhibitor depends on the respective cell-line [137].

1.3.5 The TGFß signaling pathway and VEGF

TGFß (mainly TGFß-1) is an important regulator of VEGF-secretion by folliculo-stellate cells. Normally, VEGF is solely produced by follciulo-stellate cells and not by any of the hormone-secreting cells [141]. However, VEGF-production has been shown in lactosomatotroph GH3, corticotroph AtT20 and human pituitary tumor cells thus suggesting that hormone-secreting cells can acquire the ability of VEGF-production via transdifferentiation into tumor cells [141]. Vascular endothelial growth factor is an important proangiogenetic factor, inducing the proliferation of endothelial cells and neovascularization. Autocrine secretion of VEGF has been shown in various tumors and elevated levels were found in many tumor patients ([142, 143]). Via its proangiogenetic effect VEGF contributes to survival of proliferating tumor cells, since otherwise they might suffer from hypoxia leading to necrosis [142]. VEGF levels increase during hypoxia, mediated via increasing levels of hypoxia- inducible- factor 1 (HIF1)[144]. The expression-level of VEGF by adenoma cells suggests that VEGF is not only secreted due to new blood vessel formation [144]. Furthermore, VEGF targets tumor cells specifically, interfering with and inducing various intracellular pathways, which makes their role in tumorigenesis even more complex than previously thought. What is more, VEGF does also interact with various immune cells, who express different VEGF-receptors, mainly leading to immunosuppression. It does so by impairing immunopoiesis and by inhibiting effector-functions of mature immune cells [142]. For instance, VEGF suppresses T-lymphocyte infiltration through inhibition of the NFκB signaling pathway [145], and inhibits the maturation of dendritic cells [142]. This way, authors concluded, tumor-cells escape immune-response thus promoting tumor formation and resilience.

The hypophysis is a highly vascularized organ. VEGF-production in the healthy adenohypophysis is the main task of the folliculo-stellate cells, and TGFβ-signaling plays an essential role in its regulation [144]. More recently Corlan et al. could also demonstrate VEGF-overexpression within human folliculo-stellate cells [146]. Folliculo-stellate cells enhance blood vessel formation in the pituitary gland, though other cell-types within the hypophysis seem generally able to produce VEGF themselves [144, 147]. Fetal-liver kinase 1, a VEGF-receptor subtype, seems to play an important role in pituitary tumorigenesis [144, 148].

Apart from hypoxia multiple signaling pathways, endocrine and exocrine factors lead to a balanced VEGF-production. If this balance is disrupted, tumor formation might occur. and TGFβ-3-isoforms have a stimulatory effect on VEGF. Still, TGFβ-1 and -3 are not the only factors which direct VEGF production. For instance, pituitary adenylate cyclase activating peptide and IL-6 stimulate, dexamethasone suppresses VEGF-release via glucocorticoide receptors expressed in FS cells [141, 149]. Also, estrogen influences the TGFβ-1/TGFβ-3 – ratio, which induces lactotroph hyperplasia, possibly leading to elevated Prolactin-serum levels in pregnant and lactating women [141]. Consequently, this could also be a possible mechanism involved in prolactinoma development [141]. What is more, VEGF production on lactotroph cells within the pituitary gland was found to be stimulated by estradiol in a dose-dependent manner [150]. Especially Prolactinoma and non-functioning pituitary adenoma show a high VEGF- expression level [148]. This accounts especially for invasive macroprolactinoma and NFPA, as for invasive and aggressive pituitary adenoma in general [72]. However, most pituitary tumors show low microvascular density (MVD) as compared to healthy pituitary tissue, even invasive and aggressive PA [146, 151, 152]. A small percentage of metastatic pituitary carcinoma showed high MVD [153]. Still, VEGF overexpression was generally reported in pituitary adenoma by various authors [146].

1.3.6 Differences in sex of the TGF signaling pathway mediated by dopamine and estradiol contributing to different pituitary tumor manifestations and outcome

TGF β- signaling in the anterior pituitary has been described by various authors. Dopamine-agonists and estradiol, which are responsible for lactotroph function / prolactin-secretion in the pituitary gland, were demonstrated to regulate TGF β-synthesis activation itself as well as various other components in the signaling pathway within the pituitary gland [154]. At least in part, the regulating function of estradiol and dopamine on lactotrophic function seems to be mediated by the TGF β- signaling [154]. DA lead to an increase in TGFβ-1 synthesis, estradiol to a decrease in TGFβ-2 receptor expression [154].

Dopamine is well known as a main inhibitor of lactotrophic function via dopamine type 2 receptors (Drd2) [155]. On the other hand, estradiol acts as the main stimulus for prolactin-secretion, once by inhibiting dopamine-release in the hypothalamus, and by directly stimulatory effects on proliferation and secretion of pituitary lactotrophs [155].

Still, various other cytokines and signaling pathways are regulating these processes. Also, it has to be taken into consideration, that effects observed in animal-models might not be transferable on human pituitaries. For instance, high estrogen levels demonstrated little effect on prolactin secretion in human pituitaries, as compared to rats, where a correlation was demonstrated [155]. It was suggested, that estradiol might be exerting additional distinct effects on other intra-pituitary growth factors, contributing or inhibiting tumor formation [155].

Gender-specific differences in pituitary tumor incidence, size and behavior are well-known [155]. Prolactinoma prevalence is significantly higher in women during the fertile time (20-50 years of age), than in men. During this period the female/ male- ratio lies in between 10:1, with the frequency equaling after the fifth decade [155]. The higher incidence of microprolactinoma in women as compared to men has mainly been attributed to the delay in diagnosis for men. Women develop more specific symptoms such as secondary amenorrhoea as compared to men, male sexual dysfunction or loss of libido are most often underestimated as being rather unspecific [155].

Interestingly, sex differences in the expression levels of the distinct components of the TGFβ signaling pathway superfamily, including also BMP-4, have been demonstrated [154]. Reduced expression-levels of TGFβ-1 were suggested to play a role in the development of female prolactinoma in the hCG β positive female pituitary, but not in male pituitaries [154]. Due to that, diagnosis is often not made until mass effects occur. Still, various authors suggest that the delay of diagnosis cannot explain other observations: Prolactinoma in men tend to be more aggressive, are showing higher proliferative indices and surgical treatment is often not curable [155].

In other pituitary- tumor types sex differences in prevalence and tumor behavior have been observed. ACTH-secreting pituitary adenoma occur again more often in women, but show a more aggressive behavior with higher rates of invasion and reoccurrence in men [155]. No differences have been observed in somatotropine secreting adenoma [155].

For Non-functioning pituitary adenoma, which are the focus in this work, prevalence rates of both sexes are the same. However, macro – NFPA are more common for men at an older age than for women [155]. Gender differences in the regulation of the TGF β-1 system correlate with prevalence rates of prolactinoma [155].

1.3.7 The TGFß signaling pathway, EMT, fibrosis and DARP

Epithelial-mesenchymal transition (EMT) describes a process, by which an epithelial cell undergoes biochemical changes giving it the mesenchymal cell phenotype. For this, the cell has to give up its cell polarity and intercellular adhesion. It gains, on the other hand, the key features of a mesenchymal stem cell, including enhanced migratory capacity, invasiveness, resistance to apoptosis and increased production of ECM components [156]. Once the basement membrane disintegrates, a mesenchymal cell can migrate away from the epithelium and the transition is completed [132]. Various intra- and extracellular processes are involved in the initiation of EMT – one important factor involved is TGF β. EMT is most likely mainly a phenotypical, plastic process, as is highlighted by its reverse process, the mesenchymal-epithelial-transition (MET) [156]. EMT occurs in three biologically distinct settings:

- EMT-type 1, occurring in association with implantation, embryogenesis and organ development
- EMT-type 2 associated with inflammation-induced tissue regeneration and fibrosis
- EMT-type 3 associated with tumor growth, invasiveness and metastasis [132].

On the surface, the outcome is the same. However, the involved mechanisms, on extra-, inter- and intracellular scale, vary dramatically dependent on the setting [132]. In analogy to epithelial cells, endothelial cells forming the blood vessel can also contribute to fibrosis via endothelial- mesenchymal transition [139], which has been illustrated by experiments concerning cardiac fibrosis. EndMT is also a known source of cancer-associated fibroblasts (CAFs), facilitating tumor progression (Zeisberg et al., 2007, cited by [139]. It is estimated that up to 40% CAFs derive from endothelial cells [139].

TGFβ seems to be a key regulator of EndMT, EMT-type 2 and type 3, as has been confirmed by many authors [132, 139] - TGFβ is expressed in various types of tumors and also a known mediator of EndMT (Nakajima et al., 2000, cited by [139]). EMT induction by TGFβ works via both a Smad2/3- dependent pathway, and a MAPK-dependent-pathway [157]. The Smad-mediated EMT-processes mainly facilitate cell motility. Modulatory function of inhibitory-Smads in relevant transcription factors are thought to induce the autocrine function of TGFβ, further reinforcing the EMT-Program [132]. A schematic illustration of induced EMT is shown in Fig.7.

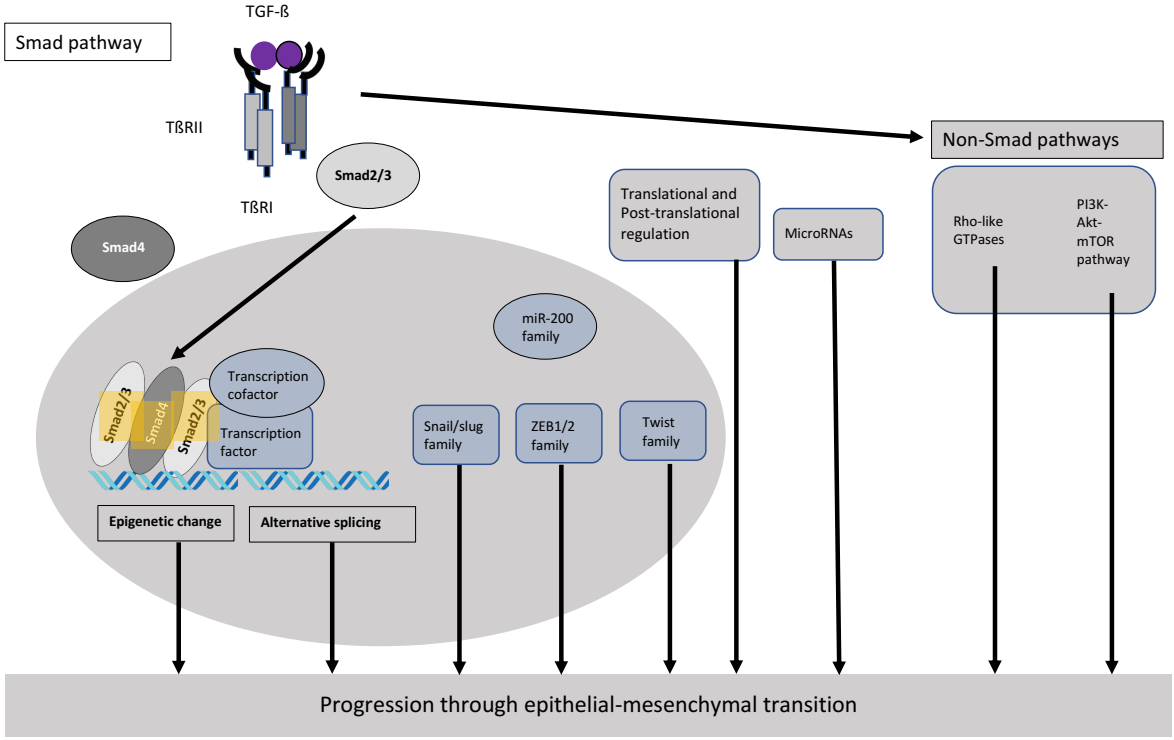


Figure 7: Schematic illustration of the Smad- dependent and Smad independent TGFβ induced epithelial-to mesenchymal transition and the genes/ transcription factors involved, adapted from Katsuno et al., 2013 [5]

The resulting fibrosis out of EMT-type 2 regulated by TGFβ has been illustrated by many authors in various experimental settings. For instance, continuous injections of TGFβ-1 lead to fibrosis in many organs, such as the liver and the kidney [158]. Application of human recombinant BMP-7, a known antagonist of TGFβ-induced EMT, could reverse fibrosis in mouse-kidney-cells in a dose-dependent manner [159].

Bin Hu, Zhigang Mao et al. demonstrated that TGFβ-1 induces type I and III collagen expression. What is more, the authors could show, that SB431542, a known inhibitor of this signaling pathway, led to a reduction in fibrosis accordingly [158]. SB431542 is also used as a TGFβ-1- pathway inhibitor in this work.

Clinical observations have been made, which showed that partial- dopamine- resistant prolactinoma have a higher rate of fibrosis as compared to sensitive prolactinoma [158]. Thus, the potential role of fibrotic remodeling in the development of drug resistance has been investigated. Since an upregulation of the TGFβ-1/Smad3 – pathway has been shown in DARPs as well, a relation between fibrosis and activation of this pathway has been examined.

1.3.8 The Role of the TGFß signaling pathway in NFPA

Various authors have confirmed that the TGFβ signaling pathway plays an essential role in development of NFPA as well as other pituitary tumors, especially those with particular aggressive and invasive features that show resistance to conventional therapy options. There is still some controversy over the role of the TGFβ signaling pathway in pituitary tumors and whether it acts as a promotor or inhibitor of tumor growth. Some authors found a significant downregulation of TGFβ/Smad signaling cascade in DARPS [7]. On the other hand, Zhenye et al. could demonstrate that levels of Smad7, an inhibitory-Smad, gradually increased the more invasive/aggressive the NFPA became. This was accompanied by a significant downregulation of SMAD3 and TGFβ-levels in NFPA. Thus the authors concluded that TGFβ acts as a suppressor in the development of NFPA and this role is tarnished in NFPAs [5]. Also, it has been shown that the down-regulation of TGFβRII expression correlates with tumor growth and invasion in non-functioning pituitary adenomas [64]. The downregulation of the TGFβ signaling pathway has been suggested to be used as a molecular marker in a predictive model for NFPA regrowth and prognosis by Cheng et al. (2019), besides the methylation status of the promotors for CDKN2A/p16, WIF1 and two clinical signatures [160]. Combined, the predictive accuracy for tumor regrowth was 81,2% [160].

1.4 Aim of this Study

With my work I hope to accomplish a further understanding of the role of the TGFβ signaling pathway in the development of pituitary tumors, focusing on non-functioning pituitary tumors. By testing established inhibitors of the aforementioned pathway, I hope to give an outlook on possible new treatment options, especially for non-functioning pituitary adenoma, which are difficult to treat especially if transsphenoidal surgery is not successful or not possible.

For that, different established inhibitors of the TGFβ signaling pathway on cell viability and VEGF-secretion pattern of mouse-model cell lines and human pituitary tumor samples were tested, focusing on Smad-signaling. I also tested expression levels of TGFβR I/II, TGFβ-1/-2/-3, Smad2/3/4-mRNA on our mouse cell-models via Northern Blot.

2. Materials and Methods

2.1 Chemicals

2.2.1 Culture media, buffer, diluents

Table 5: Culture media used

Cell culture medium	Dulbecco´s modified Eagle´s medium (pH 7,3) 10% fetal calf serum, (CFS) 2,2/l NaHCO3, 10mM HEPES 2mmol/l L-glutamine 105 U/l penicillin/streptomycin 2.5 ng/l amphotericine B.
Wash buffer	200µl 0,05% 20 Tween in PBS
Reagent Diluent	1% BSA in PBS

2.2.2 Inhibitors of the TGFβ signaling pathway

Pan-neutralizing antibodies of TGFβ and TFβRII have already been investigated by *in vivo* mouse models as well as in few preclinical studies. Surprisingly, no obvious tumor-promoting, inflammatory or otherwise adverse effects could be observed *in vivo.* Invasion and migration of the tumors could be significantly reduced (Robertson et al. cited by [137]). Still, considering the multiple effects of the TGFβ signaling pathway within various cell-types, the complete suppression of the pathway as a potential treatment option is critical. Directly targeting the specific components altered within the tumor might be more promising. For this, however, the specific alterations within the individual tumors which lead to malignant transformation have to be investigated and thoroughly understood.

In order to specifically target the imbalanced pathway components resulting in tumor formation without tarnishing the tumor suppressive effects of the TGFβ signaling pathways various potential small-molecule inhibitors of the pathway have been introduced. Many of them have already shown promising results as antitumor-agents in human cancers.

SB-431542: SB-431542 is a specific inhibitor of TGFβ superfamily type I receptors ALK4, ALK5 and ALK7 [137]. It did not interfere with other intracellular pathways activated by TGFβ, such as the ERK, JNK, or MAPK pathways (Robertson et al. cited by [137]). It has been studied within various tumor types where the TGFβ signaling

pathway has been involved showing promising results. However, as with the TGFß signaling pathway itself, the effect of SB-431542 is dual, dependent upon the respective tumor-cell-line. In some tumors it led to promoting, in others to suppressive effects [137]. For this reason, the effect of SB-431542 must be individually examined in order to predict the potential as an anti-tumor agent. This applies logically for those tumors in which the tumor-promoting effects of the TGFβ-signaling pathway predominate [137].

SIS3: SIS3 is a well- known selective inhibitor of Smad3-phosphorylation, triggered by TGFβ-1 and thus the DNA-binding ability of Smad3 [65]. It also decreased phosphorylation of constitutively endogenous active Smad3 probably by interaction with the SSXS motif located at the carboxyl-terminus of the DNA-binding site MH1 [65]. In a scleroderma-fibroblast cell-model, 3μM of SIS3 could reduce phosphorylation and DNA-binding ability of Smad3 by 50% [65]. This inhibitory effect of SIS-3 was however abrogated when SMAD3- binding sites were mutated. Phosphorylation of other Smad-proteins, as well as other TGFß induced pathways (MAPK/p38, ERK) was not altered by SIS3 [65]. Also, cell-number as well as GAPDH mRNA levels remained unaffected in dermal-fibroblast cell-models. In this model, TGFß and Smad3 were directly involved in procollagen synthesis [65]. These findings are in concordance with the results of other authors. For instance, Yamazaki et al. could show that high levels of Smad3 correlated with the process of epithelial-mesenchymal transition (EMT) in pancreatic ductal adenocarcinoma [7]. In these cells, Smad3 reduced E-cadherin expression and vimetin upregulation resulting in increased invasion [7]. Smad3 knockdown reduced the EMT-like features of these cells. High levels of Smad3 also correlated with shorter survival times of PDAC-patients [7].

In other tumors, however, contrary results could be seen. For instance, in MEN1, an autosomal-dominant disorder with an inactivating mutation of the tumor-suppressor gene menin [63]. MEN1 is characterized by an increased susceptibility to endocrine tumors of the parathyroids, pancreatic islets and the anterior pituitary [63]. As was shown by Hiroshi Kaji et al., Menin inhibited the tumor-suppressive, anti-proliferative effects of TGFβ-1 [63]. In this study Menin showed a close interaction with Smad3, but not with the other Smads. Menin significantly inhibited Smad3 dependent transcription by interacting with its MH2 domain [63]. The authors admitted however that Menin may also block effects of corepressors, allowing TGFβ- signaling [63].

Furthermore Wolfraim et al. could show that reduced Smad3 expression resulted in T-cell leukemogenesis [161]. Chunhui Liu et al also demonstrated that low levels of Smad3 and p-Smad3 protein were correlated with invasion of NFPAs [24].

Taken together these findings underline the fact that the role of TGFß in tumorigenesis is dual. The effect of Smad3 as tumor- promoting or suppressive might also be cell-type specific. It is however clear that Smad3 is an essential mediator in endocrine tumors, especially non-functioning pituitary adenoma formation. For this reason, we used SIS-3 in our study in order to determine the effects of SIS-3 in gonadotrope cell models as well as in human NFPA- cells, which are derived from gonadotrope cells and are possibly affected by unbalanced Smad3.

Cordycepin: Cordycepin (3´-desoxyadenosine) is isolated from the *fungus cordycepis militans*, which is used in complementary medicine. It has also been recognized by basic research as an effective anti-tumorigenic drug, due to its antiangiogenic, antimetastatic and anti-proliferative effect. Cordycepin has also shown promising results by some in vivo cancer-models. Cordycepin, however, shows cytotoxic effects in various ways, e.g. by suppression of mitochondrial activity [162]. Still, as was demonstrated by Wang et al. Cordycepin also suppressed TGFβ- induced EMT in an ovarian-cancer- stem cell model [162].

Due to its cytotoxic effect by various intracellular mechanisms we used Cordycepin as a control in some of our experiments in order to complement our results.

2.2 Cell lines and primary cell cultures

2.2.1 Cell Culture of Mouse-Model Cell Lines TtT-GF and αT3-1

Mouse- pituitary cell models

αT3-1-cells: Non-functioning pituitary tumors are heterogenous in origin [10], which makes it difficult to choose suitable cell-models. Before NFPA could be assigned to a specific cell-type by transcription factors it has already been documented in many cases that NFPA show α-Subunit production, common of glycoprotein-hormones, though no clinical evidence of hormonal hypersecretion could be determined [62]. Still, gonadotropinoma make up the majority of NFPA (around three quarters). Therefore, we focused our experiments of this large subtype of NFPA. We found the αT3-1-cell-line to be the most suitable for our experiments as they represent the characteristic features of gonadotropinoma, as they represent an established gonadotrope model

cell line. Cells were first transformed, obtained and immortalized by Windle et al., 1990 via genetic targeting of tumorigenesis. The authors directed expression of Simian-virus-40 (SV40) T-antigen (Tag) oncogene to pituitary cells, targeting the promoter/enhancer region from the human glycoprotein α-subunit gene [68]. Founder transgenic mice that expressed the fusion-gene developed pituitary tumors, from which the cells were extracted and put into culture [68]. The established cell-line expresses, synthesizes and secretes endogenous mouse α-subunit but no β-subunit. There are two cell-lines in the anterior pituitary which express glycoprotein hormones, namely thyreotropes (TSH) and gonadotropes (LH/FSH). These hormones share a common α-subunit but express a different hormone-specific β-subunit (LHβ, FSHβ- and TSHβ). As Windle et al. could show, αT3-1-cells do express GnrH-receptors and react to GnRH with α-subunit expression rising, but not to TRH. They do not express and secrete β-subunit and consequently no functioning complete LH/FSH. Thus, authors concluded that the cells represent gonadotrope precursor cells and can be distinguished from thyreotropes by the lack of TRH-responsiveness [68].

TtT-GF-cells: The mouse TtT/GF-cell line has been widely used as a model- cell line of folliculo-stellate cells [14], which are involved in the regulation of various endocrine functions of the pituitary gland. Furthermore, various studies have hinted that these cells are able to transdifferentiate, which raises the possibility that these cells do also represent a pituitary stem cell population [14]. For our study, however, we used the Tt/GF-cells mainly as a positive control of VEGF-production and proliferation in response to TGFβ-1/-3 and the respective inhibitors.

We compared the results obtained from these animal-model cell types with parallel experiments with human NFPA primary cell cultures.

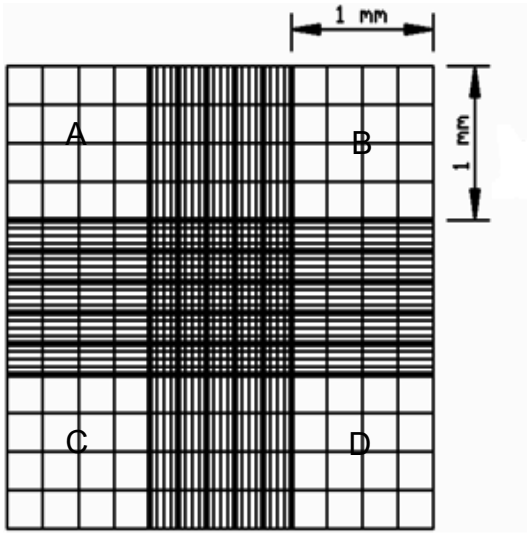
The folliculo-stellate-like TtT/GF mouse pituitary tumor cell line was a gift of Prof. Kinji Inoue from the Saitama University, Urawa, Japan, and gonadotroph-like nonsecreting αT3-1 mouse pituitary tumor cells were kindly provided by Prof. Pamela Mellon, University of California, San Diego, La Jolla, USA.

The cell lines were kept in aliquots in liquid nitrogen at -179° C for long-time storage. For thawing, the tubes were warmed up to 37 °C in the water bath and the cell suspension (1ml) was diluted with 20 ml culture medium (see below). This was followed by a centrifugation with 1200 rpm for 3 min at room temperature. The supernatant was discarded and the pellet re-suspended in fresh culture medium.

Cells were initially seeded into 25 cm^2 flasks and were cultivated in an incubator at 37 °C, 95 % air humidity and 5 % CO_2 concentration. When the cells had reached confluence, they were transferred to 75 cm^2 flasks in which they were continuously kept for 30 to 40 passages.

For both the TtT-GF and αT3-1 cell lines Dulbecco´s modified Eagle´s medium (pH 7,3) was used. The medium was supplemented with 10% fetal calf serum, 2,2/l NaHCO3, 10mM HEPES, 2mmol/l L-glutamine, 105 U/l penicillin/streptomycin, and 2.5 ng/l amphotericin B. The flasks were kept in a CO_2 -incubator at 37°C at 95 % air humidity and 5 % CO_2 concentration. Any procedures involving living cells were performed under strictly sterile conditions using a laminar flow cabinet. As for any adherent cell culture, cell passaging was performed every 3-4 days depending on cell density, remaining surface area and color of the medium. The state of the cells was evaluated regularly by inspection under an inverted microscope. For passaging/splitting of the cells the old medium was first aspirated. In order to get rid of remaining cell detritus 15ml of PBS was added and subsequently aspirated in a washing step. Then, 4 ml Trypsin/EDTA (Sigma) solution was applied in order to detach the adherent cells from the flask. After an incubation time of 3-4 minutes cells detached from the flask´s surface, which could be visually controlled under the inverted microscope. The detachment could be accelerated mechanically by softly shaking the flask. As soon as sufficient floating cells could be visualized 10ml of culture medium was added in order to stop the adverse trypsinization-induced effects on the viability of the cells. The cell suspension was then transferred into a 50 ml tube and centrifuged with 1200 rpm at room temperature for 3 min. After the supernatant was removed, the cell pellet was re- suspended using 10 ml of fresh, pre-warmed culture medium.

Then, the number of living cells was determined in a Neubauer chamber by dye exclusion test. For this 50µl of the suspended cells were mixed with 50µl of 0,4% trypan-blue solution in PBS. The blue dye cannot cross the cell membrane of living cells which therefore appear clear under the microscope. In contrast, dead cells accumulate the dye and are blue colored. The Trypan-blue containing cell- suspension was filled into a Neubauer counting chamber and put under a binocular microscope. Viable cells in 4 x 16 big squares were counted and divided by 4 to get the average cell numbers of 1 x 16 squares. The number of cells achieved in this way was multiplied with 2 x 10^4 to determine the average numbers of cells per milliliter. The principle of cell counting using the Neubauer chamber is explained in the legend of Fig. 8.



$$\text{Concentration} = \frac{\text{Number of cells x 10 000}}{\text{Number of squares x dilution}}$$

Figure 8: Neubauer chamber for cell counting. After sample preparation 10µl of the dilution is introduced into the Neubauer chamber under a glass cover using a micropipette. The loaded chamber is put under a microscope at 100x magnification. The number of viable cells (cells with green color under UV-light) was determined by the cells overlying 4 x 1 mm2 areas of the counting chamber. In order to calculate cell concentration the total cells counted in 4 mm2 were divided by 4, leading to the average cell number in 1 mm2 area. This number was then divided by the dilution factor (1:2). Multiplied with 10-4 ml, the volume of 1 mm2 area, led to the estimated cell number/ml.

Depending on the estimated cell count the cell suspension was split onto new flasks or diluted appropriately in order to achieve the cellular count needed for the planned assays, plating them on 48 - or 96-well plates respectively. Regarding multiwall plates, only the inner wells were used for seeding cells; the rows of outer wells were filled with the corresponding amount of PBS.

As soon as the passaging was accomplished the newly planted cells were covered with the respective plastic lids and put back into the incubator.

2.2.2 Primary Cell Cultures of Human Pituitary Adenoma Cells

The clinical neuroendocrinology group receives tissue of human pituitary adenomas obtained by transsphenoidal surgery from different Neurosurgical Clinics: Universitätsklinikum Erlangen; Klinikum Großhadern of the University of Munich; Klinikum Rechts der Isar of the Technical University of Munich. The studies with the tissues were approved by the local ethics committee. The patients were informed about the usage of the extracted tumor prior to the surgery and informed written consent was received from each patient.

Pituitary adenomas are usually extirpated in small pieces via curettage. If suitable samples could be extricated, they were transferred directly into a tube containing cell culture medium.

The tissue samples kindly donated to us were then sent to the laboratory. Usually, the preparation of primary cell cultures of the pituitary tissue samples could be started 1-2 days after excision.

As PAs are typically highly vascularized tumors it is rather difficult to gather pure tumor cell samples from the extricated tissue. For this reason, some parts (e.g. blood clots) had to be removed from the tissue sample in a petri dish using a scalpel. The tissue had to be washed several times with HDB+-buffer in order to eliminate remaining further blood contamination. Then the tumor tissue was cut with a scalpel into small pieces and appropriate portions were transferred to 15-ml tubes containing 5 ml collagenase-mix. The tubes were put into a water-bath at 37 °C for about 60 to 90 minutes, depending on the consistency of the tumor tissue. Every 15 minutes the tubes were retrieved and the tissue suspension was gently pipetted multiple times with Pasteur pipettes under the sterile workbench to accelerate the loosening of the cell-cell contacts mechanically. When the cell suspension was homogeneous and no pieces of tissue could be observed by eye examination, the enzymatic dispersion was stopped by adding 10ml of culture medium. The cell suspension was then centrifuged at 1200rpm for 4 minutes at room temperature. The supernatant was discarded and the pellet was re-suspended in 10ml of culture medium. The centrifugation was repeated one more time and cells were re-suspended again. In order to loosen the cell pellet from the bottom of the tube the suspension was carefully pipetted up and down multiple times. Counting of human pituitary adenoma was performed after acridine orange/ethidium bromide staining which in comparison to the trypan-blue method (see above) allows a better discrimination between living and dead cells. To this end, 50 µl cell suspension was mixed with 50 µl acridine orange/ethidium bromide solution (50 µl acridine orange (200 mg/l) + 50 µl ethidium bromide (10 mg/l) + 900 µl PBS) and counted in a Neubauer chamber as described above. Under UV-light, living cells appeared in green and dead cells in red in the microscope. As human pituitary adenoma cells proliferate extremely slowly, the cells were dissolved with cell culture medium to achieve a density of 200 000 cells per ml and 0.5 ml of this cell suspension were directly seeded into the inner wells of 48-multiwell plates (corresponding to

100 000 cells per well); 0.5 ml PBS was put in the outer layer of wells of the plate. Experiments were performed as soon as the cells had attached to the plastic surface. Cells were exposed to different-conditions, TGFß-1, 3 or/ and with different inhibitors known to interfere with the TGFß signaling pathway. Following treatment, cell viability was measured using different established assays (WST-Assay, 3 ^{3}H-thymidine Incorporation-Assay). Furthermore, VEGF levels in response to exposure to the different agents were determined using mVEGF-ELISA. The same procedure was done to different cell-cultures derived from our human tumor-cell-samples, that were obtained from transsphenoidal surgery.

The findings were analyzed using Statistical procedures. RNA-expression of the different SMADs was shown by Northern-Blot. After that, results of the different cell-lines and tumor-samples were compared, looking out for parallels as well as significant changes in behavior following treatment to the different conditions.

2.3 Methods

2.3.1 Measurement of Cell Proliferation

In order to analyze the effect of TGFβ and the respective inhibitors of its pathway on cell proliferation and viability, several assays with distinct principle designs exist, which all have different advantages and disadvantages. Generally, 4 distinct cell-proliferation assay designs exist (67, see also table below).

In this study, two cell proliferation assays with distinct concepts were used: (1) The WST-1 Assay, which measures metabolic activity correlating with viability and proliferation rate of the cells. (2) The ^{3}H-thymidine Incorporation Assay which measures DNA-replication rate using the radioactive nucleotide analogue ^{3}H-thymidine as a marker.

Table 6: Overview about established methods for the determination of cell proliferation and viability (derived from Yadav et al. 2001 [163]

Concept	Examples	Advantages	Disadvantages
Measurement of DNA-replication	^{3}H-thymidine- and BrdU-based assays	Precision, DNA-synthesis as more direct marker of mitosis as compared to cell viability/metabolic rate	Radioactivity for ^{3}H-thymidine assay, special laboratory equipment needed.
Analysis of metabolic activity	WST-1, MTT, XTT, resazurin,	No radioactivity, relatively cheap.	Cell viability and metabolic rate do not always directly correlate with cell proliferation.
Variations of ATP-concentrations	Different-kit-based assays by different companies, no generalized protocol	Very sensitive for small number of cells; fast, as no incubation step is needed.	Short half-life of light emission, so illuminators with reagent injectors to extend the signal are needed. Not sensitive enough for cells with low proliferation rate.
Cell-surface antigen-based assays (cell-proliferation markers)	Ki-67, topoisomerase IIB, phosphor-histone H3 and PCNA	Cell-surface antigens as very sensitive markers for proliferating cells.	Changes in proliferation with cells with low basic proliferation rate are hardly detected.

2.3.1.1 ^{3}H-thymidine- Incorporation Assay

Slowly growing human adenoma cells were seeded in 48-well plates with a density of 10^5 cells per well, whereas the initial number of rapidly proliferating TtT-GF and αT3-1 cells was 10^4 cells per well (in some cases 0.5 x 10^3). After 24 h, when the cells had attached to the plastic surface of the multiwall plates, they were treated with different concentrations of stimulating and inhibiting agents, alone or in combination, for different treatment periods (24-72 h), as indicated in detail in the “Results” section. During the treatment time the cells were placed in the incubator at 37 °C, 5% CO_2 and 95 % humidity.

In case of slowly growing human primary adenoma cell cultures, 3H-thymidine (0.5 µCi per well) was added to the wells at the beginning of the treatment period, as due to the low cell division rate only a small amount of 3H-thymidine was incorporated into the adenoma cells. In case of the rapidly growing pituitary tumor cell lines, 3H-thymidine was added to the wells only during the last 4 h of the treatment period. At the end of the treatment period, the cell culture supernatant was removed and 0.5 µl ice cold 10% TCA solution (in PBS) was added to each well to fix the cells at the plastic surface and the cell culture multiwall plates were incubated for 1 h on ice in the refrigerator at 4 °C. Then, the TCA solution was discarded and 500 µl of a mix of 0.1% Triton and 0.5 M NaOH (100 ml PBS + 2g NaOH + 100µl Triton X-100) was put into the wells to destroy the cells and to hydrolize the DNA with the incorporated 3H-thymidine. After overnight incubation at room temperature, the cell culture plates were shaken for about 5 min and the content of the wells, containing all tritium that was integrated into cellular DNA, was transferred to a 5 ml scintillation vial. Next, 4 ml of Ultima Gold scintillation cocktail (Packard Bioscience, Groningen, The Netherlands) were added, the vial was closed and carefully shaken to get a homogeneous emulsion of scintillation cocktail and tritium-containing Triton/NaOH mix. The vial was then put in a β-counter (LS 6000 IC; Bibby Scientific, Staffordshire, UK) and the number of scintillations was measured as counts per minute (cpm).

2.3.1.2 WST-1-Assay

The WST-1 procedure is an established cell viability assay we used it as an alternative for comparing cell proliferation of the TtT-GF- and αT3-1-cell lines in response to the treatment with the respective inhibitors under different conditions. The WST-1 assay was not used for primary human pituitary adenoma cell cultures as for slowly growing cells this assay is not sensitive enough.

The WST-1-Assay is a metabolic-activity based assay. The ratios of metabolites such as NADH/NAD (nicotinamide adenine dinucleotide), FADH/FAD (flavin adenine dinucleotide), FMNH/FMN (flavin mononucleotide) and ATP concentrations all increase corresponding to cellular proliferation rate (67). These metabolic intermediates reduce tetrazolium salts to a formazan product via dehydrogenases/ reductases. The reduction potential of the proliferating cells can be detected by the resulting colorimetric / fluorescent change using a spectrophotometer or microplate reader (67). WST-1 is an water soluble tetrazolium salt. It is negatively charged and

thus does not readily penetrate cells, unlike the positively charged viability markers such as MTT (67).

The general steps of the WST-1-Assay are as follows [163]:

1) Cells are cultured until reaching an appropriate cell count.
2) The tested mediators/ inhibitors are added.
3) Incubation at 37°C for varying incubation periods depending on the experiment.
4) Addition of WST-1- solution at concentrations of about 0,33 mg/ml/well.
5) Incubation for another short period ranging in between 10min- 2h depending on the tested cell line and cellular concentration. During incubation, cells have to be kept in the dark using e.g. aluminum foil containers.
6) Absorbance levels are measured at 420-480nm in a microplate reader.

Cells were cultured and plated at different cellular concentrations measured by Neubauer counting chamber. After varying incubation periods with our tested mediators/ inhibitors ranging in between 24h- 72h.Then, 10 µl of WST-1 (Roche®) was added to each well and incubated at 37°C under 7% CO_2 in a humidified incubator for variable time periods (20min- 4h). The WST-1 agent was added under appropriate sterile conditions under a laboratory work bench. Before adding the WST-1 agent, the aliquot was protected from light using aluminum foil and the light under the bench was switched off to minimize destruction of the compound by light. 10µl of WST-1 was added to each well and as soon as possible, cells were covered using a dark box in order to protect them from light. At the end of the WST-1 incubation period, the absorbance levels were measured in a microplate reader (Bio-Rad) at 450/655 nm. In a series of preliminary experiments I found that the optimum incubation period with the WST-1 reagent was between 10 and 20 min for Tt-GF cells, while it was about 3h for the αT3-1 cell line.

2.3.2 Measurement of VEGF production

Cellular expression levels of VEGF were measured with an ELISA Kit following the principle of a sandwich ELISA. For the human tumor samples the human VEGF-ELISA Kit (R&D Systems, MN, USA), was used, for the mouse TtT-GF/ αT3-1-cell lines the mouse VEGF-Kit accordingly from the same manufacturer. Measurements were done following the manufacturer´s instructions. The amounts of the respective

agents/solutions, however, had to be adjusted to the smaller sized plates. For that half the amount per well of the respective reagent was used.

Enzyme-Linked Immunosorbent Assay (ELISA) is an established technique to detect and quantify specific amounts of a target antigen in samples such as blood, urine and cell culture supernatant. The so-called “Sandwich-ELISA”, which measures the amount of antigen between two layers of antibodies (capture and detection antibody) is more sensitive and specific compared to other ELISA methods. The principle design of VEGF-ELISA is shown in figure 9. The general steps are as follows:

- A known quantity of Capture Antibody is coated within polystyrene plates
- Binding sites left open within the plates are blocked by e.g. BSA-PBS solution. (Bovine-Serum albumin binds to the remaining spots nonspecifically).
- The samples are added to the microplates together with a series of diluted standards with a known quantity of the target antigen as reference.
- Unbound antigens are washed from the plate
- A biotinylated Detection Antibody is added, which binds specifically to the target antigen.
- Plates are washed again, in order to remove unbound Detection Antibodies.
- A complex of Streptavidin with Horse-radish peroxidase (Streptavidin-HRP) is added.
- Unbound Streptavidin-HRP is removed in another washing step
- The Substrate Solution (1:1 mixture of H2O2 and Tetramethylbenzidine) is added. The Substrate is converted by HRP into a color.
- The enzymatic process is stopped after a given time period by addition of e.g. sulfuric acid.
- Absorbency of the wells can be measured using a multi-well spectrophotometer set to determine the presence and quantity of the antigen. The diluted standards with known concentrations of the target antigen serve as reference in a standard curve.

The exact protocol offered by R&D Systems for the mVEGF- and hVEGF-ELISA-Kit was as follows:

- Fresh 96-well ELISA plates were filled with 50 µl anti-mouse/human VEGF antibody (Capture Antibody, CA) per well at a dilution of 1 µg/ml in PBS solution. Plates were covered with Parafilm® and left to incubate overnight.
- The next day wells were washed three times with 200 µl of Wash Buffer (200 µl 0,05% 20 Tween in PBS) using a multichannel pipette. By tapping the plates upside down on fresh paper towels remaining liquid within the wells could be removed. At this point the proper fixation of the mVEGF-

capture antibodies (CA) onto the surface of the plates could be anticipated. In order to saturate the remaining CA 150 µl of Reagent Diluent (1%BSA in PBS) per well was added, followed by an incubation time of 1 h at room temperature. All antibodies with the exception of the Capture Antibody were diluted in Reagent Diluent.

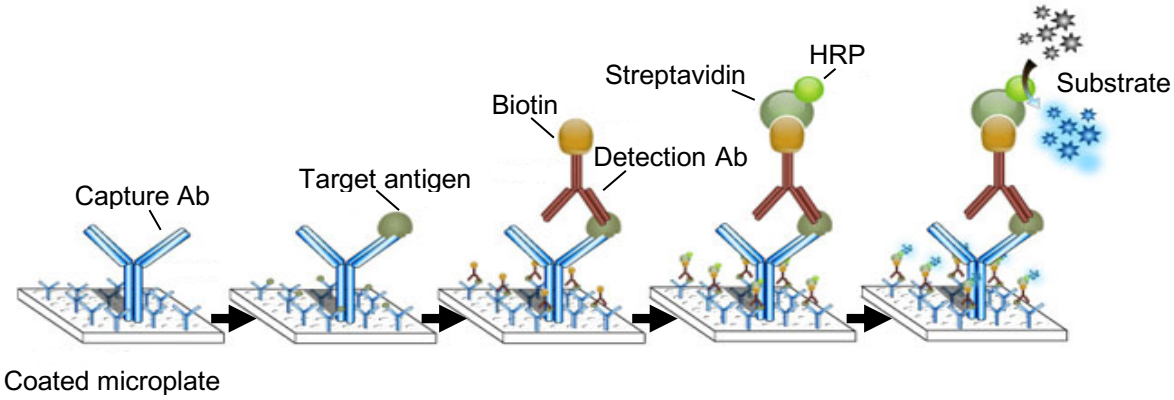


Figure 9: Principle design of Sandwich-ELISA; 96-well ELISA polystyrene microplates are coated with a known quantity of Capture Antibody (CA), here against VEGF as the target antigen. After the binding of the CA with the target antigen another antibody is added: The Detection antibody forms a complex with the target antigen (here VEGF) at another binding site. The Detection antibody is biotinylated. Streptavidin conjugated with horse-radish peroxidase (HRP) binds to the Biotin on the Detection Antibody. Then a so-called Substrate solution is applied. The horse-radish peroxidase enzymatically converts the substrate (here 1:1 mixture of H2O2 and Tetramethylbenzidine) into a color (here blue). By addition of the so called “Stop solution” (here: sulfuric acid) the enzymatic conversion is blocked after a given incubation period. Finally, the absorbency of the individual plates can be measured using a multi-well spectrophotometer set to 450nm to determine the presence and concentration of the antigen. The absorbency measured is compared to a standard series/ standard curve as reference.

After saturation of the remaining enzymes plates were washed again for three times with washing buffer as was described before. Seven mVEGF standards with concentrations ranging from 15,6pg/ml to 1000pg/ml were prepared. For hVEGF measurements standard dilutions ranged from 32,2pg/ml to 2000pg/ml. Standards were added on two rows of the plates as reference. 50 µl of the thawed samples or standards were added to each well. Afterwards, plates were covered with a new Parafilm® and left to incubate for 2 h at room temperature. Another three washing steps were performed, and 50µl of a primarily prepared Detection-Antibody solution (100ng/ml) was added to each well respectively. Plates were incubated again for 2h and then washed as was described before. Afterwards 50µl of Streptavidin-HRP (5µl/ml) per well was added. In order to avoid interference by sunlight plates were covered with aluminum foil. After incubation of 20min at room temperature plates were washed once more for three times. 50µl of Substrate Solution was added and, again, left to incubate for 20min under light protection. The reaction was stopped by addition

of 25µl of 1M sulfuric acid ("Stop-Solution"). Absorption at 450 nm was measured immediately by an ELISA plate reader. Absorption values at 550 nm were subtracted to achieve higher accuracy. From the measured absorption values, the plate reader calculated the mVEGF-concentrations based on the standard curve created from the absorption values the VEGF standard values.

2.3.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Northern-blot

To study whether αT3-1 cells synthesize mRNAs of the different TGFß isoforms, receptors and relevant Smad signaling components, RT-PCR was performed to detect the corresponding mRNAs. TtT/GF cells, in which mRNA synthesis of these factors has already been demonstrated (Lohrer et al.) served as positive control.

2.3.3.1 RNA isolation

For RNA extraction and purification, TtT/GF and αT3-1 cells were grown in culture medium in 6-well plates until they had reached confluence corresponding to about $1x10^6$ cells per well. The cell culture medium was removed and the cells were washed twice with PBS. Then cell lysis was performed by adding 0.5 ml TRIzol reagent (Invitrogen) per well to the cells, which were mechanically removed from the plastic surface using a cell scraper. The cell lysate was homogenized by pipetting up and down with an insulin syringe. RNA isolation from the lysate was then performed according to the TRIzol manufacturer's instructions. At the end the amount of isolated RNA was measured with a UV-photometer (Nano Drop 1000™, Thermo Scientific). To this end an appropriate aliquot of the RNA sample was diluted with RNAse-free DEPC water and was put into the photometer and the absorbance was measured at 260 nm (nucleic acid absorption maximum) and at 280 nm (protein absorption maximum). In this way the purity of the RNA sample could be approved and a 260/280 absorbance ratio of >1.8 was considered pure. To calculate the amount of RNA the following formula was used:

Absorbance 260 x sample dilution x 40 = µg RNA/ml

As the RNA sample may still be contaminated with DNA, PCR (see below) with a house keeping gene (GAPDH) was performed and in case of DNA contamination, the RNA sample was treated with DNAse I.

2.3.3.2 Reverse Transcription

As the extracted RNA sample contains a mix of different types of RNA, the mRNA has next to be transcribed into complementary DNA (cDNA). For reverse transcription 1µg RNA, 1 µl of dNTP mix (2 mM), 2 µl of 62.5 U/ml random primers (Hexanucleotide mix), 2 µl Dithiothreitol (DTT, 10 mM), 1 µl of 200 U reverse transcriptase (SuperScript II), 4 µl of 5x first strand buffer were mixed and filled up with DEPC water to a final volume of 20 µl. This mixture was then incubated for 1 h at 45. The reaction, during which the cDNA was produced, was stopped by boiling the sample at 95 ºC for 5 min. The cDNA sample was then used for PCR.

2.3.3.3 Polymerase Chain Reaction

As I aimed to prove only the expression – but not the precise amount – of mRNA of TGFß, its receptors and Smad signaling components, only semi-quantitative PCR was performed. The primers specific for the detection of the different TGFß related compounds are listed in Table YY. For the PCR reaction 1 µl of the cDNA samples obtained from αT3-1 and TtT/GF cells were mixed with 1.5 µl10x PCR buffer, 0.9 µl $MgCl_2$ 25 mM, 1.5 µl dNTP mix 2 mM, 0.5 µl amplification primer sense 10 pmol/µl, 0.5 µl amplification primer antisense 10 pmol/µl, 0.15 µl thermua aquaticus (Taq) DNA polymerase and 8.95 µl sterile distilled water. The PCR reaction in a thermo block heater consisted of 35 cycles each containing the following steps: Denaturation for 1 min at 94 ºC, annealing for 30 sec at 58 ºC and PCR fragment elongation fo 1 min at 72 ºC.

2.3.3.4 Gel electrophoresis (Northern Blot)

The amplified fragments were separated by electrophoresis using ethidium bromide agarose gel in 1 x TBE buffer for 15 to 20 min at 80 V and then visualized under UV light.

Table 7: Primers used for the detection of RNA of TGFß, TGFß receptors and Smad signaling components

Target§	Primer sequence (5´⟶3´)	cDNA size
TGF-ß1	5´: GCT GCG CTT GCA GAG ATT AAA 3´: TTG CTG TAC TGT GTG TCC AG	552 bp
TGF-ß2	5´: CCA AAG ACT TAA CAT CTC CCA CC 3´: GTT CGA TCT TGG GCG TAT TTC	684 bp
TGF-ß3	5´: GCT CTT CCA GAT ACT TCG AC 3´: AGC AGT TCT CCT CCA GGT TG	440 bp
TGF-ßR1	5´: ATC CAT CAC TAG ATC GCC CT 3´: CGA TGG ATC AGA AGG TAC AAG A	824 bp
TGF-ßR2	5´: CGT GTG GAG GAA GAA CAA CA 3´: TCT CAA ACT GCT CTG AGG TG	560 bp
Smad2	5´: CGG AGA TTC TAA CAG AAC TG 3´: TGC TTG AGC ATC GCA CTG AA	846 bp
Smad3	5´: AGC ACA CAA TAA CTT GGA CC 3´: TAA GAC ACA CTG GAA CAG CGG ATG	636 bp
Smad4	5´: CAT TCC TGT GGC TTC CAC AA 3´: GAC TGA TGG CTG GAG CTA TT	803 bp

§ specific for the detection of the corresponding mouse RNA

2.5 Statistics

All experiments with the cell lines were repeated at least 3 times and performed in quadruplicate which means that 4 wells in a cell culture multiwell-plate had the same conditions.

Studies with human pituitary adenoma cells were dependent on the amount of cells obtained from the tumor tissue and not all tests for stimulating and inhibiting agents - alone or in combination - could be performed.

Calculations and statistics were performed with Microsoft Excel and SPSS. Values are shown as mean ± standard error and analysis of variance (ANOVA) in combination with Scheffe's test was used to determine statistical significance and p-values smaller than 0.5 were considered significant.

3. Results

3.1 Detection of the TGFβ signaling pathway in pituitary tumor cell lines

In a previous study by Renner et al. it has already been shown, that folliculo-stellate-like TtT/GF cells expressed all components of the TGFβ signaling pathway, whereas it has not yet been investigated whether this is also the case for nonfunctioning αT3-1 cells. By semiquantitative PCR and subsequent blotting of the amplified cDNA I could not only confirm mRNA synthesis of all three isoforms of TGFß (1, 2, 3), of the two TGFβ receptors (TGFβR1, TGFβR2) and of the intracellular TGFβ signaling proteins SMAD 2, 3 and 4 in TtT/GF cells but could also detect the mRNA synthesis of all these components in αT3-1 cells (see fig. 10).

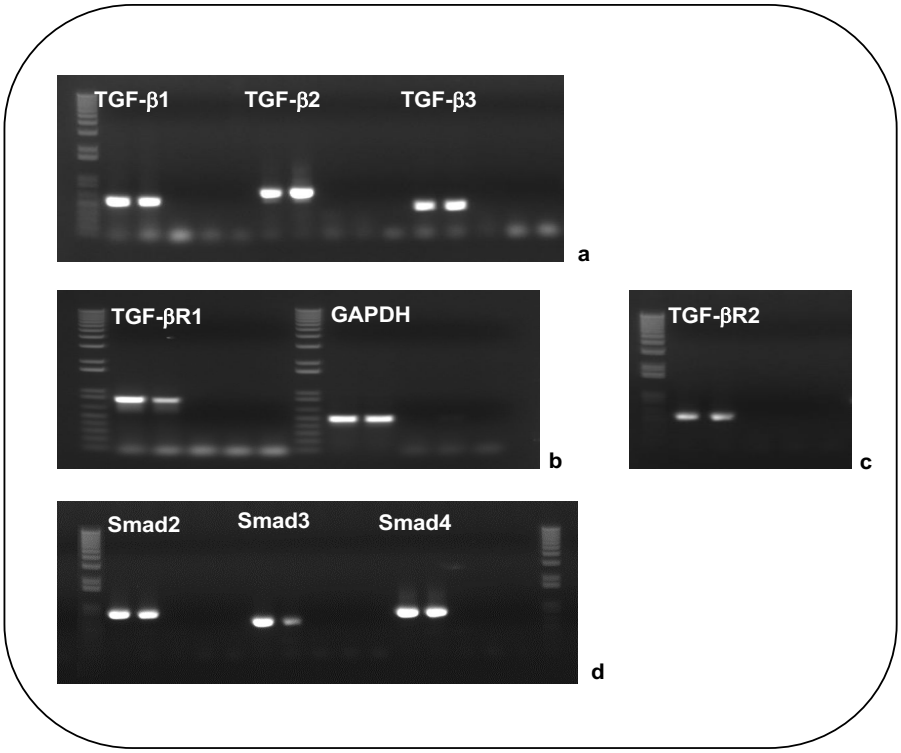


Figure 10: Detection of mRNA of TGF-β isoforms, TGF-β receptors and SMAD signaling proteins. Left bands represent aT3-1, right bands TtT-GF-cells. GAPDH(b) = glycerin-aldehyde-3-phosphate-dehydrogenase, loading control. As only qualitative PCR assay was performed band intensities do not correspond with expression levels. a: mRNAo fall three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) are expressed in both TtT-GF and aT3-1-celllines. b,c: TGF-β receptor1 and 2 are expressed in both celllines as well. d: mRNA of relevant Smad signaling proteins (Smad2, Smad3, Smad4) in extracts of aT3-1cells (left bands) and TtT/GF cells (right bands)

The expression of not only TGFβ receptors and subsequent signaling proteins but also of the TGFβ isoforms itself suggests that TGFβ may be produced by the cell lines and

may thus affect growth and function of αT3-1 and TtT/GF cells in autocrine manner (see also chapter 4).

3.2 Effects of TGFβ isoforms and TGFβ inhibitors on the proliferation of mouse pituitary tumor cell lines

In order to investigate the effects of TGFß-1 and TGFβ–3 and the different inhibitors on cell growth in the mouse cell lines I first tested the capability of two different assays, the non-radioactive WST-1 assay and the ^{3}H-thymidine incorporation assay, which are both well-established assays to investigate cell proliferation. After plating the cells were stimulated with varying concentrations of TGFβ-1, TGFß-3, SIS3 and SB431542 alone or in combination. In some experiments, the inhibitory effects of curcumin on basal and/or TGFß-1 or TGFß-3 stimulated growth were also studied. I performed both dose-response studies with varying doses of the stimulators and inhibitors as indicated and time course studies stimulating the cells for 24, 48 or 72h with the different compounds.

3.2.1 Proliferation studies with the WST-1-assay

For the WST-1 assay we plated the cells at a concentration of 2500 and 5000 cells/well for relatively large TtT/GF cells whereas 5000 and 10000 cells/well of the small αT3-1 cells were seeded into 96-well plates. As the cells were growing very rapidly, lower cell numbers were found to be more appropriate for growth experiments, as in the wells with the higher seeding numbers, cells formed confluent monolayer, in particular after 48 and 72 h treatment periods, and then stopped to proliferate further. The WST-1 proliferation assay is based on the assumption, that higher numbers of cells have higher amounts of metabolic enzymes converting the WST-1 reagent. Thus, in principle, untreated cells will have a certain metabolic activity to convert WST-1 which will result in a specific absorbance value whereas cells in which the cell proliferation has been stimulated or inhibited will have higher or lower absorbance values, respectively. As the cell culture medium itself had a small measurable absorbance at 450 nm, on each multi-well culture plate 4 to 6 wells had to be filled with medium alone and the measured absorbance background value had to be subtracted from absorbance values determined in the wells containing proliferating culture cells. Importantly, before starting with measuring cell proliferation with the WST-1 assay, the optimum duration for incubating the cells with the WST-1 reagent has to be determined as the different cell types have different metabolic enzyme rates which is critical for the

formation of the colored formazan product, whose absorbance is measured. For TtT/GF cells, an incubation period with the WST-1 reagent for 20 min had already been established in the lab. In a series of experiments, I found out that αT3-1 cells had to be incubated for 3 h with WST-1 reagent to deliver optimum absorbance values.

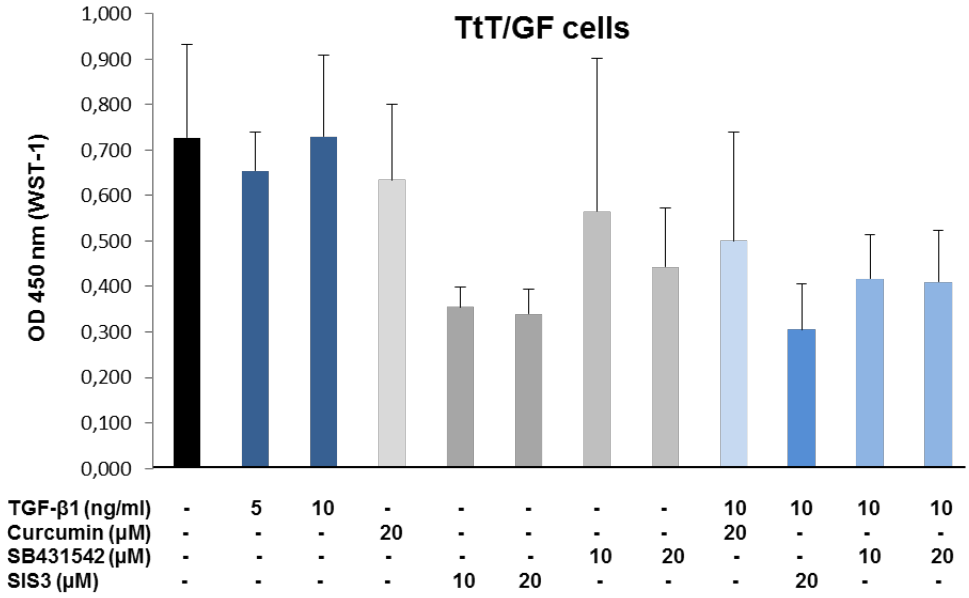


Figure 11: Representative results from proliferation experiments with the TtT/GF cell line using the WST-1 assay; similar results were also obtained with αT3-1 cells. The high variability of the quadruplicate values leading to high standard errors for each treatment condition made it difficult or impossible to detect significant differences among the various treatment conditions although some trends were obvious suggesting growth suppression of the cell lines by the different inhibitors.

However, in a series of experiments the WST-1-assay often delivered inconsistent inhibitory or stimulatory results under the same experimental settings and moreover, the quadruplicate measures showed a high level of variance leading to values with high standard errors which made it impossible to detect significant differences between the treatment conditions (see figure 11). We concluded that the assay was less suitable to study effects of TGFß and TGFß inhibitors in pituitary tumor cell lines for reasons that will be discussed below.

3.2.2 Proliferation studies with the 3H-tymidine incorporation assay

With the ^{3}H-thymidine incorporation assay only dividing cells are measured and no interferences with other cellular processes disturb the measurements. This makes this assay a robust and reliable tool to determine cell growth. Based on the experience with the WST-1 assay, cells were seeded at a density of 2500 cells per well (TtT/GF cells) or at 5000 cells per well (αT3-1 cells) into 48-well cell culture plates and the treatment of the cells was started 24h later when the cells had attached to the plastic surface.

Time course experiments with stimulators or inhibitors over periods of 24, 48 and 72 h showed linear stimulatory or inhibitory effects on the proliferation of αT3-1 and TtT/GF cells during this time period (data not shown). As significant differences between the applied compounds on the proliferation could already be detected after 24 h and did not further improve during longer treatment periods, in all further proliferation experiments cells were treated with the different compounds for 24 h. As the proliferation rate of both αT3-1 and TtT/GF cells was very high, 3H-thymidine was added three 4 h before the end of the 24 h treatment period to achieve a sufficient, well-measurable 3H-thymidine incorporation into the cells.

With the ^{3}H-thymidine Incorporation cell proliferation assay it could be shown that SB431542 and SIS3 dose-dependently inhibit αT3-1 and TtT/GF cell growth both in the absence and presence of TGFß-1 or TGFß-3 (Figures 12 and 13,). TGFß-1 alone had no effect on the growth of the rapidly proliferating cell lines and the same was observed for TGFß-3. The combination of TGFß-1 or TGFß-3 with the respective inhibitors also did not show less cell proliferation as the respective inhibitor alone. This suggests a constitutive active Smad signaling pathway with respect to cell proliferation (see chapter 4).

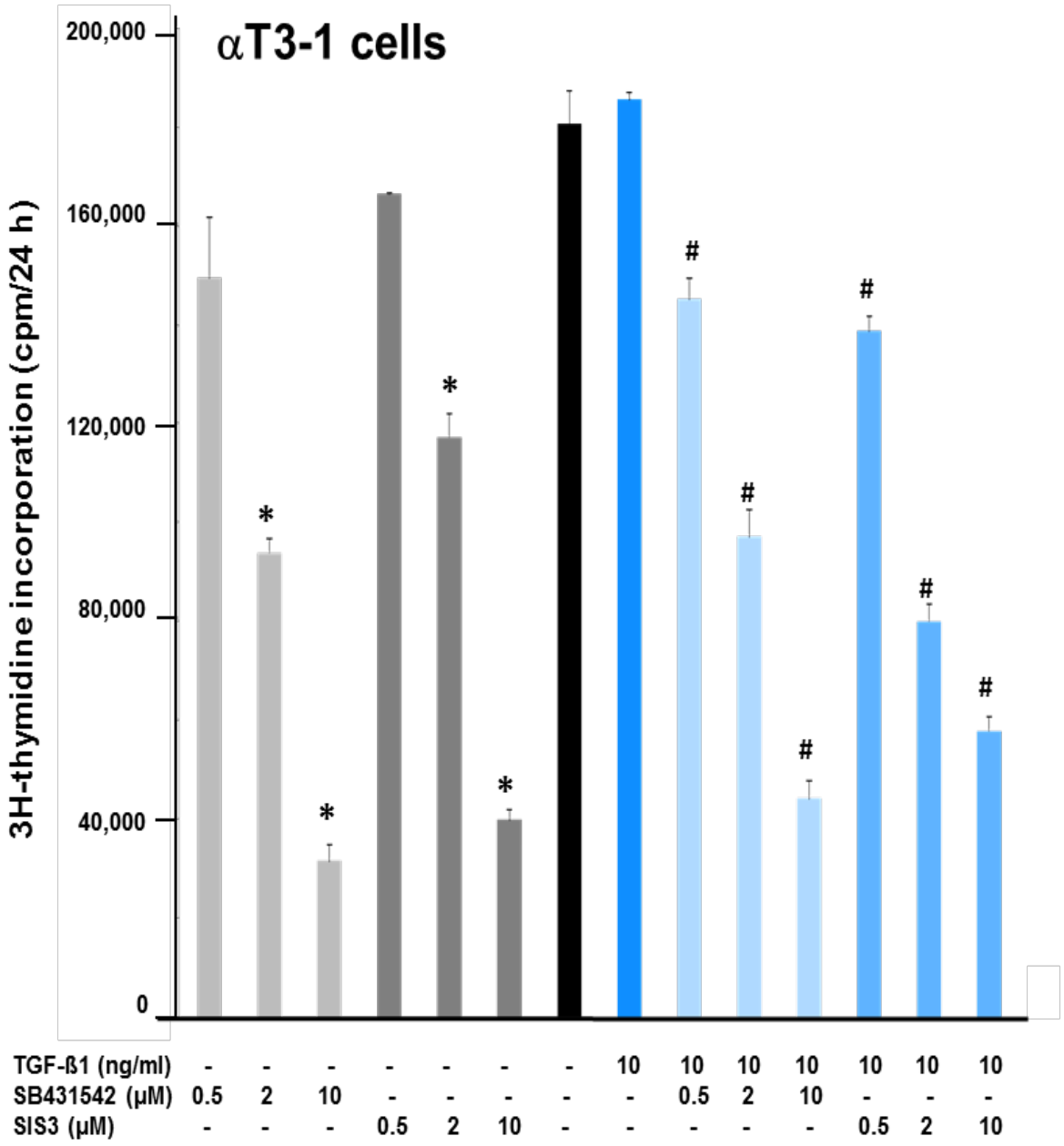


Figure 12: Effect of TGFβ–1 and TGFβ inhibitors alone or in combination on 3H-thymidine incorporation of αT3-1 cells. The application of increasing concentrations of SB431542 and SIS3 alone (grey bars) led to a dose dependent suppression of the proliferation of untreated cells (black bar). Treatment of the cells with TGFβ–1 (dark blue bar) did not further stimulate the growth of untreated cells. When added in combination, the inhbitors dose-dependently inhibited the proliferation of cells treated with TGFβ–1 (light blue bars). Cells were treated for 24 h. *p<0.05 vs. untreated cells; #p<0.05 vs. TGFβ–1 treated cells.

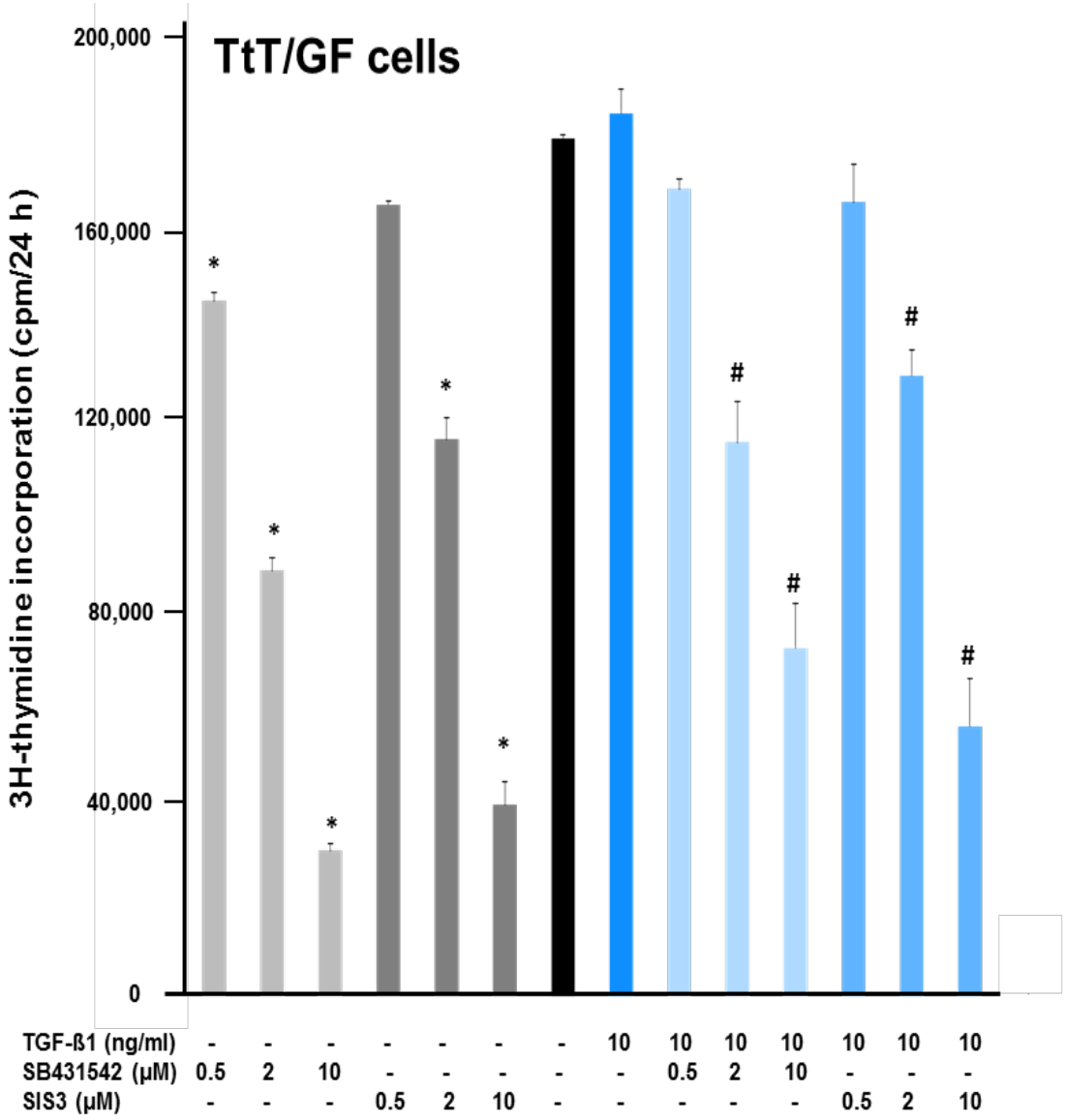


Figure 13: Effect of TGFβ–1 and TGFβ inhibitors alone or in combination on 3H-thymidine incorporation of TtT/GF cells. The proliferation of untreated cells (black bar) was suppressed by increasing concentrations of SB431542 and SIS3 (grey bars) in dose dependent manner in the absence of TGFβ–1. Treatment of the cells with the latter (dark blue bar) did not further stimulate the growth of untreated cells. When the inhibitors were added to the cells in the presence of TGFβ–1, they dose-dependently inhibited the proliferation of TGFβ–1 treated cells (light blue bars). Cells were treated for 24 h. *p<0.05 vs. untreated cells; #p<0.05 vs. TGFβ–1 treated cells.

Table 8: Effect of TGFβ inhibitors on the proliferation of TGFβ–3 treated αT3-1 and TtT/GF cells

Treatment	3H-thymidine incorporation[1] αT3-1 cells	TtT/GF cells
basal	100±9	100±6
TGF-β3 (10 ng /ml)	112±7	109±4
TGF-β3 + 0.5µM SB431542	89±5	91±9
TGF-β3 + 2µM SB431542	67±13*	72±10*
TGF-β3 + 10µM SB431542	43±8*	49±5*
TGF-β3 + 0.5µM SIS3	73±11*	87±8
TGF-β3 + 2µM SIS3	58±9*	61±4*
TGF-β3 + 10µM SIS3	39±5*	36±11*

*1) Average values of 3 independent experiments are shown. 3H-thymidine values of untreated cells (basal) were set 100% and values obtained after treatment with TGFß-3 alone or in combination with inhibitors are expressed as percent of basal. Cells were treated for 24 h. *p<0.05 vs. TGFß-3 treated cells.*

3.3 Effects of TGFβ isoforms and TGFβ inhibitors on the proliferation of human nonfunctioning pituitary adenomas (NFPAs)

During my thesis, in total 6 human nonfunctioning adenomas (designated NFPA1 to NFPA6) were available from different neurosurgical centers to study the effects of TGFβ isoforms and TGFβ inhibitors on cell proliferation with the 3H-thymidine incorporation assay. All tumors were radiologically diagnosed as macroadenomas with signs of extra- or suprasellar expansion. All adenomas were nonfunctioning tumors, as none of the patients had elevated serum hormone levels or symptoms of hormonal excess. Dependent on the amounts of cells obtained after pituitary adenoma dispersion, limited amounts of cells were available for primary tumor cell culture and thus, only limited experiments could be performed.

In primary culture, human adenoma cells grow very slowly. Therefore, after an attachment period of 24 h after seeding of the cells into 48 well plates, human adenoma cells were treated for a period of 72 h with TGFβ–1, TGFβ–3 and the inhibitors (SB431542, SIS3) alone or in combination, when possible. Due to the extremely low proliferation rate, 3H-thymidine was present during the whole treatment time of 72 h).

In tumor cell cultures from NFPA1 and NFPA2, enough cells were available to study the effects of the inhibitors alone or in combination with TGFβ–1 and - 3 (Figs. 14,15).

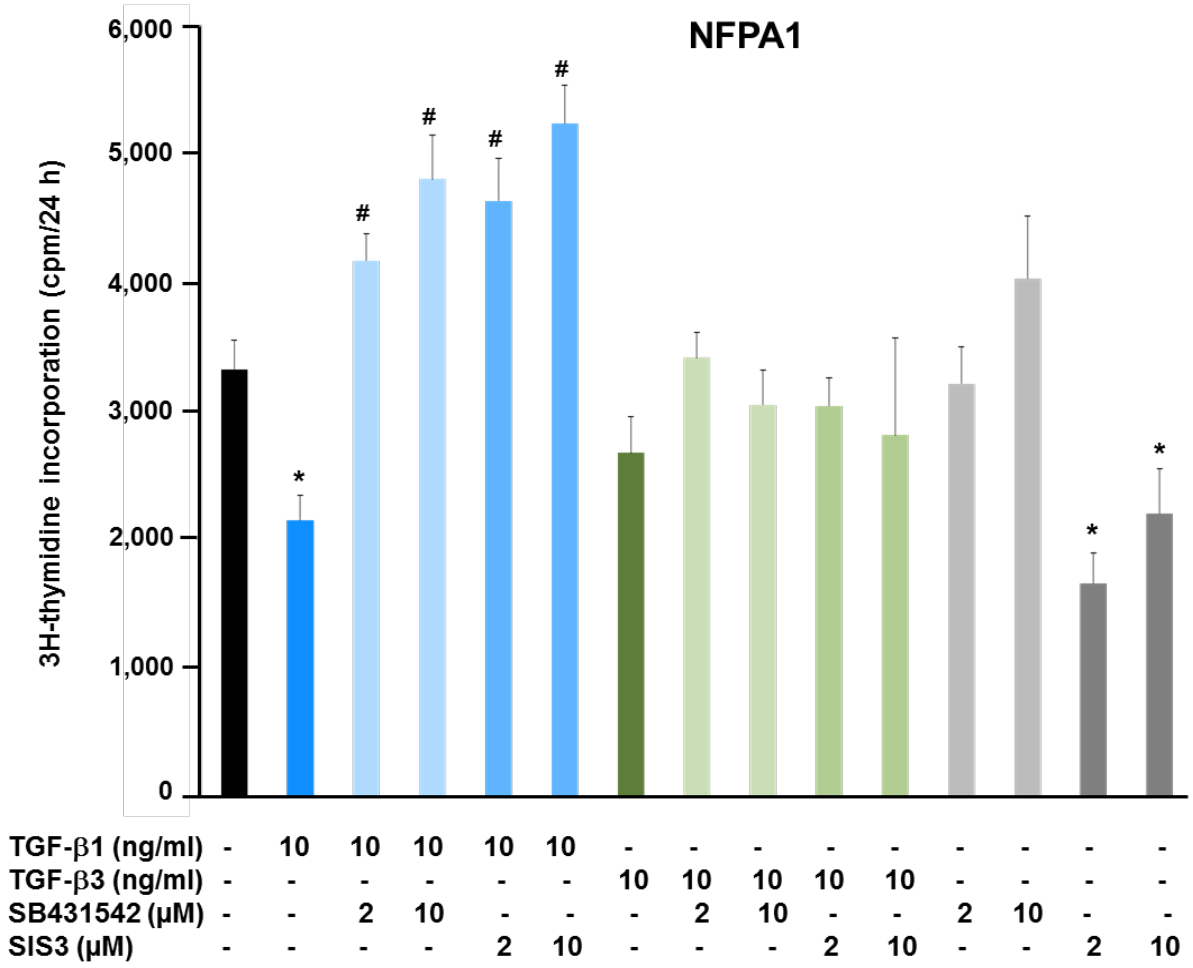


Figure 14: Effect of TGFβ isoforms and inhibitors in primary cell culture of NFPA1. In comparison to untreated cells (black bar), TGFβ–1 significantly inhibited cell proliferation and correspondingly, the SB431542 and SIS3 stimulated cell growth when added in combination with TGFβ–1 (blue bars). TGFβ–3 alone or in combination with the inhibitors had no influence on the proliferation (green bars). When cells were treated with the inhibitors alone (grey bars), only SIS3 suppressed cell growth. However, this effect decreased in the higher dose. Together with TGFβ–1 proliferation did paradoxically increase dose dependently, as was observed for SB431542. Cells were treated for 72 h). *,p<0.05 vs. untreated cells; #,p<0.05 vs. TGFβ–1 treated cells.

In these two tumors TGFβ–1 alone had opposite effects on adenoma cell proliferation and correspondingly TGFβ inhibitors had divergent effects when added together with TGFß-1. TGFβ–3 had no effect on cell proliferation (Figs.14,15)

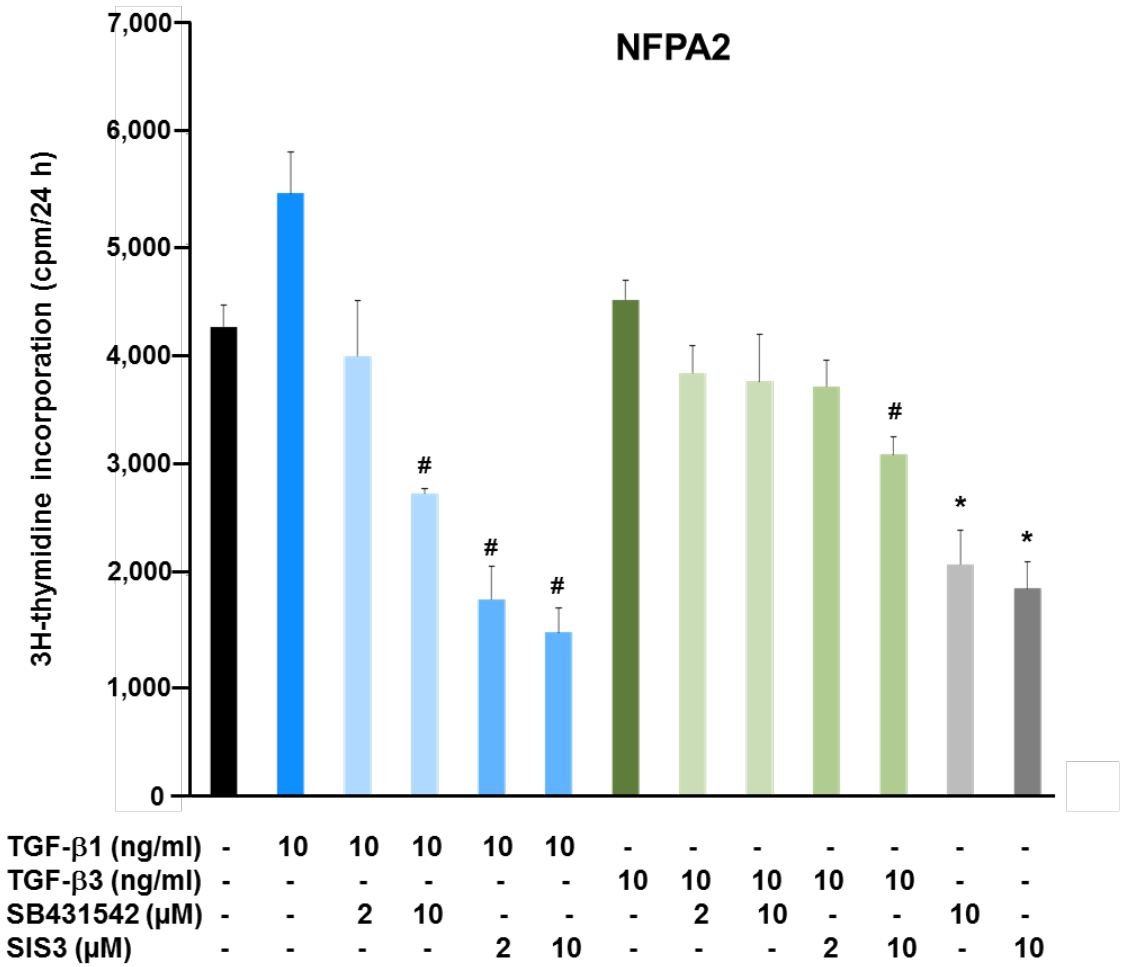


Figure 15: Effect of TGFβ isoforms and inhibitors in primary cell culture of NFPA2. In primary cell cultures of this tumor, TGFβ–1 slightly but not significantly stimulated the proliferation in comparison to untreated cells (black bar). Both SB431542 and SIS3 inhibited cell growth in the presence of TGFβ–1 and were also able to suppress cell proliferation when added alone. TGFβ–3 had no significant influence on adenoma cell proliferation but reduced the suppressive action of the inhibitors when added in combination with them. Cells were treated for 72 h. *,p<0.05 vs. untreated cells; #,p<0.05 vs. cells treated with TGFβ–1 or TGFβ–3, respectively.

In 4 additional adenoma cell cultures (NFPA3 to NFPA6; see Figure 16) only the effect of TGFβ–1 alone and in combination with the inhibitors could be tested.

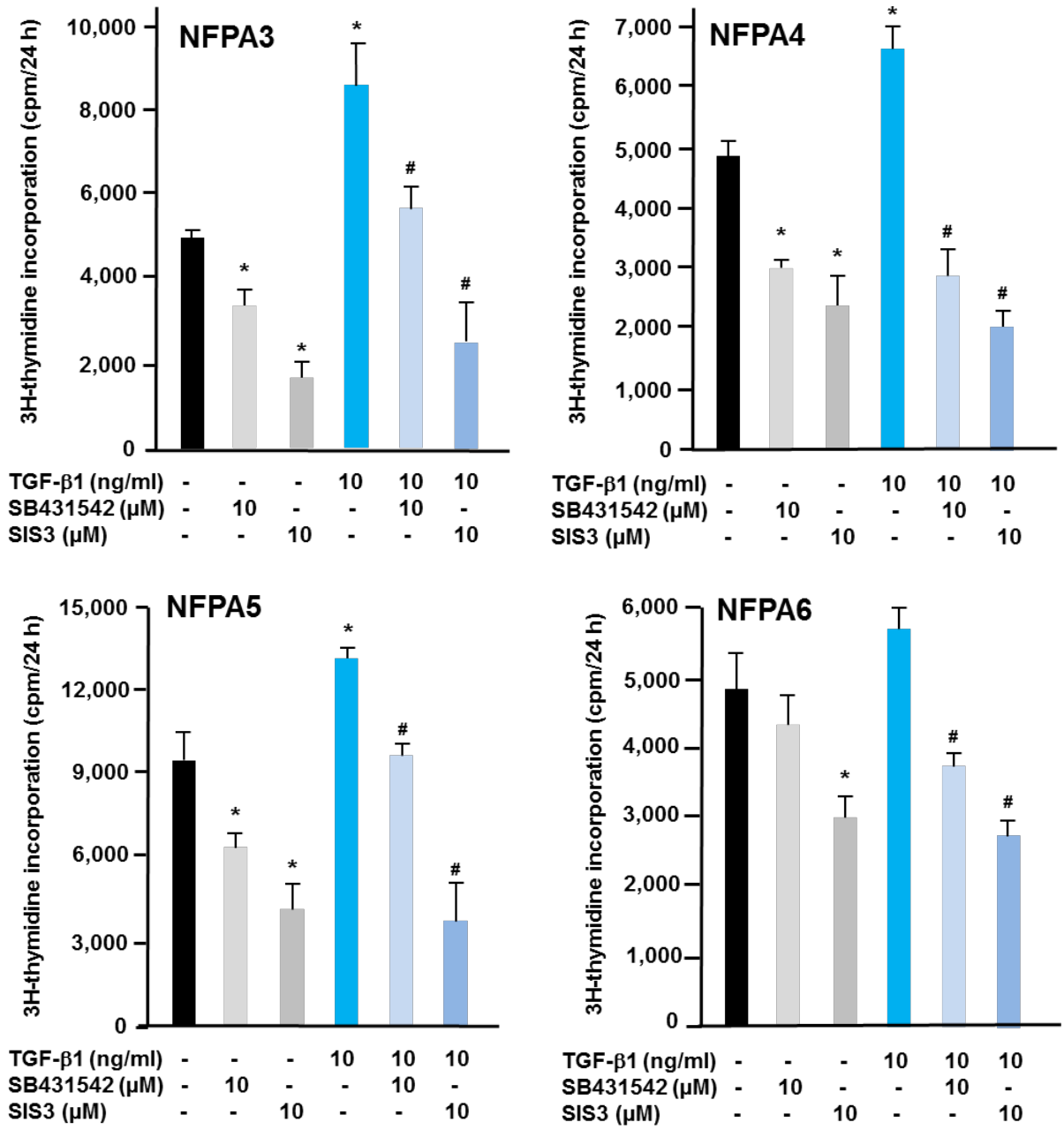


Figure 16: Effect of TGFβ–1 and inhibitors in 4 primary cell cultures of NFPAs. In three cases, TGFβ–1 significantly stimulated the proliferation of adenoma cells in comparison to untreated cells (black bar). Both SB431542 and SIS3 inhibited cell growth in the presence of TGFβ–1 and with exception of NFPA6, in which SB431542 was not effective, were also able to suppress cell proliferation when added alone. Cells were treated for 72 h. *,p<0.05 vs. untreated cells; #,p<0.05 vs. cells treated with TGFβ–1.

Thus, regarding the proliferation of the 6 human nonfunctioning pituitary adenoma cell cultures studied, TGFβ–1 had different effects on cell proliferation: In one adenoma TGFβ–1 significantly inhibited and in 3 adenomas TGFβ–1 significantly stimulated cell growth; in 2 adenomas a non-significant growth stimulatory trend could be observed. The role of TGFβ–3 could be tested only in 2 adenoma cell cultures and in none of them a stimulatory or inhibitory effect on cell proliferation could be detected. In most

cases the inhibitors suppressed the proliferation of the adenoma cells already in the absence of TGFβ isoforms. In the cell culture of NFPA1, in which TGFβ–1 inhibited the cell proliferation, the combined application of TGFβ–1 and the inhibitors led to a growth stimulatory effect. In the remaining tumor cell cultures, in which TGFβ–1 had growth inhibitory effects, the combined treatment with the inhibitors led to a suppression of cell growth. Although TGFβ–3 alone had no effect on cell proliferation, the combined application of TGFβ–3 and the inhibitors abolished the growth suppressive effects of the inhibitors. The conclusions from these findings are discussed below.

3.4 Effect of TGFß isoforms and inhibitors on the production of VEGF in αT3-1 and TtT/GF cells

TGFß isoforms may not only affect the growth of pituitary adenomas through directly affecting cell proliferation but also by suppressing angiogenesis and thus intratumoral vascularization, a process that is critical for tumor expansion. In order to approve this, the effect of TGFβ isoforms and inhibitors on the production of VEGF, a key component of angiogenesis, was studied. The investigations were only performed with αT3-1 and TtT/GF mouse pituitary tumor cells because all cells obtained from human pituitary adenomas were used for the above described proliferation experiments. For studies of the effects of TGFβ isoforms/inhibitors on VEGF production the cell lines were seeded in normal cell culture medium containing 10% fetal calf serum at densities of 10,000 cells/well (TtT/GF cells) and 20,000 cells/well (αT3-1 cells). After an attachment period of 24 h the initial culture medium was removed and replaced by a medium containing only 0.5% instead of 10% fetal calf serum. In this serum-reduced medium the cells nearly completely stop to proliferate. Then TGFβ isoforms alone or in combination with inhibitors were added and after 48 h VEGF was measured in the cell culture supernatant by ELISA as described. As under serum-reduced conditions the cells do not grow, alterations of the VEGF production by TGFβ isoforms/inhibitors are direct effects and not caused by alterations of cell numbers.

In αT3-1 cells, TGFβ–1 significantly stimulated the VEGF production (Fig. 17). Both SB431542 and SIS3 dose-dependently suppressed the VEGF release in the absence and the presence of external TGFβ–1.

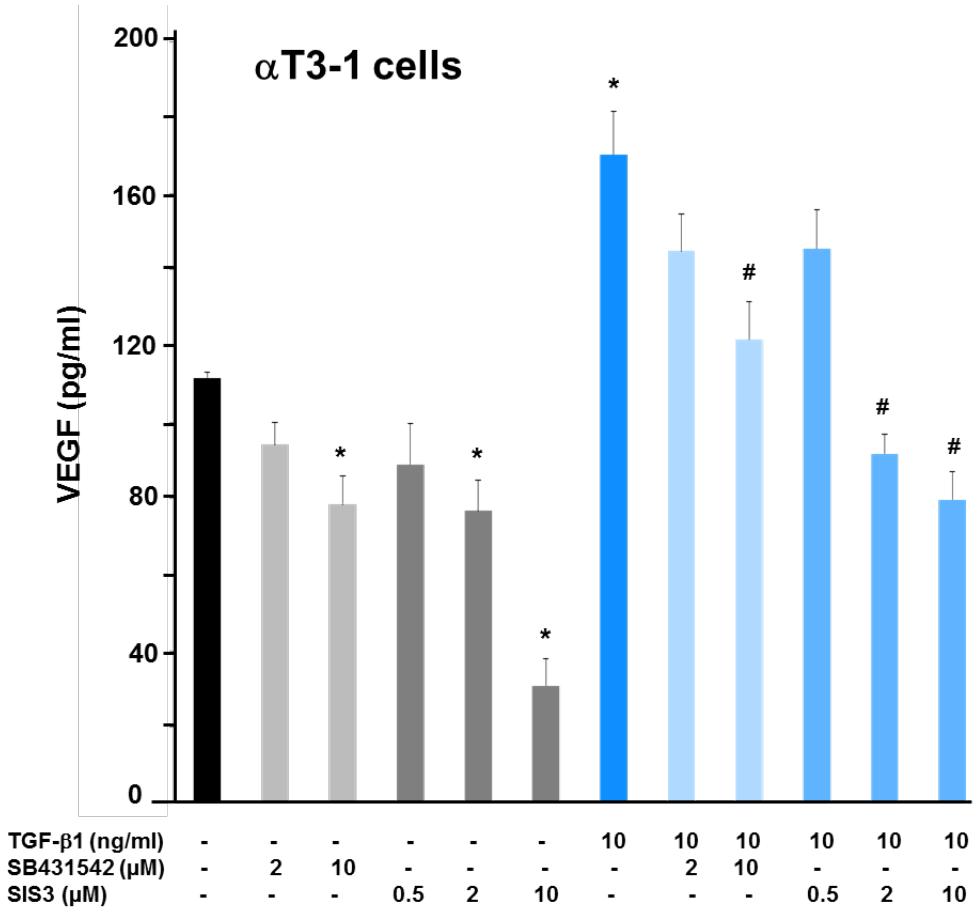


Figure 17: Effect of TGFβ–1 and TGFβ inhibitors alone or in combination on VEGF production of αT3-1 cells. The application of increasing concentrations of SB431542 and SIS3 alone (grey bars) led to a dose dependent suppression of the VEGF production of untreated cells (black bar). Treatment of the cells with TGFβ–1 (dark blue bar) stimulated VEGF production in comparison with untreated cells. When added in combination, the inhbitors dose-dependently inhibited the TGFβ–1 stimulated VEGF production (light blue bars). Cells were treated for 48 h. *p<0.05 vs. untreated cells; #p<0.05 vs. TGFβ–1 treated cells.

The VEGF production was also significantly enhanced by TGFβ–1 in TtT/GF cells (Fig. 18) and the two inhibitors suppressed the VEGF production both in the absence and the presence of TGFβ–1.

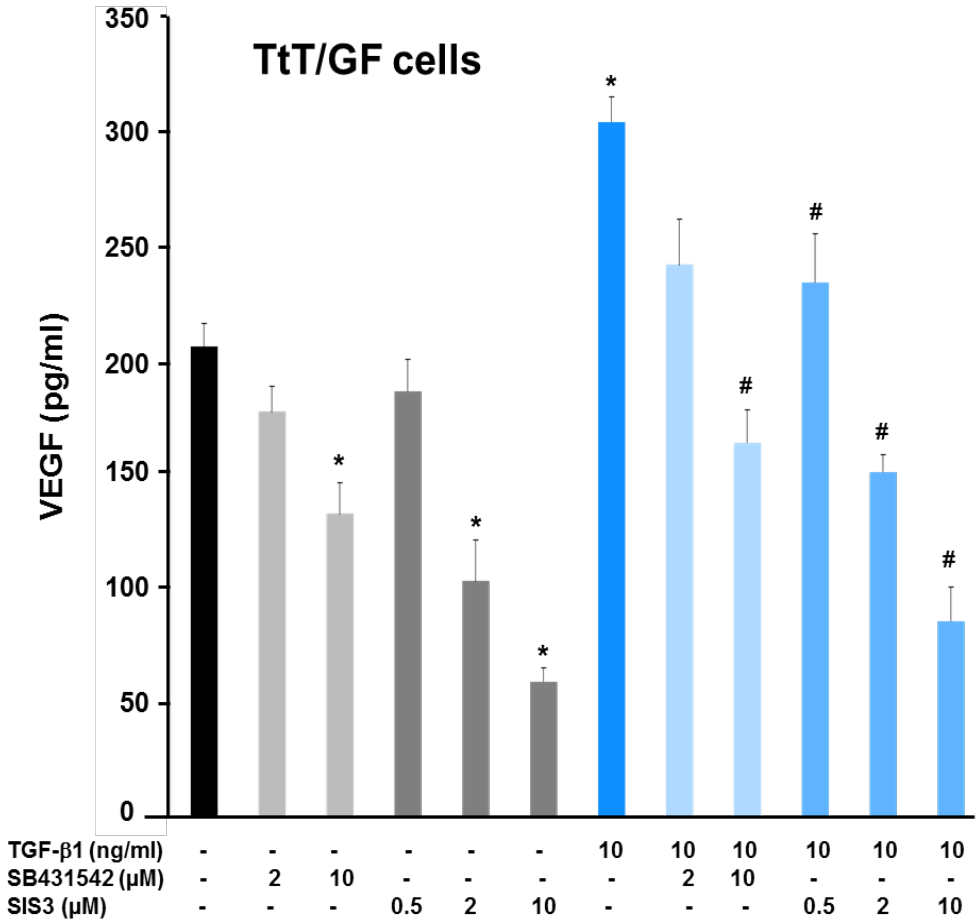


Figure 18: Effect of TGFβ–1 and TGFβ inhibitors alone or in combination on the VEGF production of TtT/GF cells. The VEGF production of untreated cells (black bar) was suppressed by increasing concentrations of SB431542 and SIS3 (grey bars) in dose dependent manner in the absence of TGFβ–1. Treatment of the cells with the latter (dark blue bar) significantly stimulated the VEGF formation. When the inhibitors were added to the cells in the presence of TGFβ–1, they dose-dependently inhibited the TGFβ–1 stimulated VEGF production (light blue bars). Cells were treated for 24 h. *p<0.05 vs. untreated cells; #p<0.05 vs. TGFβ–1 treated cells.

TGFβ–3 significantly stimulated the VEGF production of both αT3-1 and TtT/GF cells (table B). SB431542 and SIS3 dose dependently inhibited the TGFβ–3-stimulated VEGF release in both cell lines (table B)

Table 9: Effect of TGFβ inhibitors on the TGFβ-3 stimulated VEGF production in αT3-1 and TtT/GF cells

Treatment	VEGF[1]	
	αT3-1 cells	TtT/GF cells
basal	100±6	100±8
TGF-β3 (10 ng /ml)	147±12*	138±8*
TGF-β3 + 2μM SB431542	128±16	119±14
TGF-β3 + 10μM SB431542	106±11 #	88±10 #
TGF-β3 + 0.5μM SIS3	133±9	111±9 #
TGF-β3 + 2μM SIS3	108±8 #	82±6 #
TGF-β3 + 10μM SIS3	85±7 #	53±12 #

*1) Average values of 3 independent experiments are shown. VEGF values of untreated cells (basal) were set 100% and VEGF values obtained after treatment with TGF -3 alone or in combination with the inhibitors are expressed as percent of basal. Cells were treated for 48 h. *p<0.05 vs. basal; #p<0.05 vs. cells treated with TGF -3 alone.*

4 Discussion

In the present thesis I have studied the putative role of TGFß inhibitors as suppressors of the growth of hormone-inactive pituitary tumors. For the latter, so far, partial or complete cure is achieved only by surgical removal of the tumors. However, nonfunctioning adenomas are normally detected very late when they have already started to invade neighboring structures and complete removal is then not possible. Thus, pharmacological treatment concepts to prevent regrowth of incompletely resected NFPAs are urgently needed. Members of the TGFß protein family have already been discovered as triggers of the growth of different types of tumors but still little is known about the role in the development of pituitary tumors, in particular in the development of NFPAs. This may be explained by the fact that only recently TGFß-specific inhibitors like the here studied SB431542 and SIS3 have been developed that can be used as tools to block the action of TGFß and thus to investigate the role of TGFß in tumorigenesis which would then be the basis for further exploring their potential as drugs for NFPA treatment.

4.1 Discussion of the results

4.1.1 Expression of the TGFß system

NFPAs are a heterogeneous group of pituitary tumors whose common characteristic is the missing production of active anterior pituitary hormones. From previous studies it is known that most NFPAs originate from gonadotropic cells which have lost their ability to produce gonadotropic hormones during neoplastic transformation (1,11). Non-hormone producing folliculo-stellate (FS) cells make up an essential part of the tumor microenvironment (Bainott). In order to study the mRNA expression of components of the TGFß signaling pathway, I used murine αT3-1 cells, an established cell line model for gonadotroph-like NFPAs, and murine TtT/GF cells, a model of FS cell tumors. As in TtT/GF cells the mRNAs of the three TGFß isoforms (TGFß-1, -2, -3), the two receptors (TGFßRI, TGFßRII) and the relevant SMAD signaling components (Smad2, Smad3, Smad4) had already been detected, this cell line served as control for the detection of the corresponding mRNAs in αT3-1 cells. In both cell lines mRNA expression of all components of the TGFß pathway could be detected and although not studied within the frame of the present thesis, the cell lines may also produce the corresponding proteins. This is indicated by the growth and VEGF

suppressive actions of SB431542, a selective TFG- ßRI inhibitor and the inhibitory action of the Smad3 suppressor SIS3. Moreover, the stimulatory effect of TGFß-1 and – ß-3 on the VEGF production of αT3-1 and TtT/GF cells also suggests the expression of functional TGFß receptor proteins. Interestingly, both cell lines produce all three TGFß isoforms which suggests that the growth and function of these cells is not only triggered by external TGFß but may also be influenced in autocrine manner by intrinsically produced and released TGFß. However, as I have not studied, whether the cells release functional active TGFß proteins, I can only speculate about this paracrine action and more work is needed to approve this. Due to the limited amount of tissue available, it was not possible to study the expression of the TGFß system in human NFPAs. In previous studies of other authors, the expression of TGFß isoforms, their receptors and Smad proteins have already been described. My findings, that the proliferation of cells from human NFPAs was affected by TGFß and the inhibitors, suggest that - similar as in αT3-1 and TtT/GF cells - the complete TGFß system is also expressed in human NFPAs.

4.1.2 Influence of TGFß and inhibitors on proliferation

In order to avoid the usage of radioactive material with all its waste problems, I first tested the eligibility of the WST-1-assay to measure cell proliferation of αT3-1 and TtT/GF cells. The assay is based on the assumption, that cells have a distinct number of mitochondria with a certain activity of mitochondrial enzymes which change the tetrazolium salt WST-1 to formazan, which is then colorimetrically determined. An increase or decrease in cell number would thus be reflected by a rise or a decline of mitochondrial enzyme activity and correspondingly, a change in formazan formation. However, when trying to use the WST-1 assay for cell growth studies I found a high variability of the absorbance values under identical treatment conditions leading to high standard error values that made it impossible to detect significant differences between the different treatment regimens. The reason for this phenomenon may be explained by the fact, that TGFß has metabolic effects and can directly influence mitochondrial enzyme activities [164]. With the WST-1 assay changes in the proliferation of cells can only be measured when the test substances influence cell proliferation associated changes in the number of mitochondria without directly altering the intramitochondrial enzyme activity in parallel. However, as this is the case when using TGFß, the WST-1 assay is not suitable to detect TGFß induced effects on cell proliferation.

To this end the radioactive 3H-thymidine assay was finally used to study cell proliferation in αT3-1 and TtT/GF cells as well as in human NFPA cell cultures. 3H-thymidine is incorporated in the DNA of dividing cells and the changes of cell numbers are directly reflected by alterations of 3H-associated radioactivity in the cell cultures. It turned out that the 3H-thymidine assay was a robust and reliable tool to measure cell growth.

In both αT3-1 and TtT-GF cells, treatment with TGFß-1 or TGFß-3 had no significant influence on cell proliferation. This may be explained by the fact that under standard culture conditions using 10% fetal calf serum (FCS) the cell lines were growing very rapidly and may have reached maximum growth velocity. Thus, even high concentrations (10 ng/ml) of TGFß-1/- 3 would not be able to further enhance the cell proliferation. To overcome this problem, it would have been necessary to reduce the growth velocity of the cells by reducing the proportion of FCS. However, a reduction of FCS leads to a rapid decline of the growth velocity and induces a growth arrest of the cells, a fact that was used to study the effect of TGFß and its inhibitors on VEGF production. Under growth arrested conditions, TGFß isoforms had no effect on cell proliferation (see 4.1.3.) suggesting that TGFß isoforms alone may not be sufficient to reverse the cell growth arrest. Other explanations of the missing growth effects of externally applied TGFß isoforms may be that TFG- ß is present in FCS or is produced by the cells. However, the amount of growth factors in FCS, among them TGFß, is far below the TGFß concentrations used in the present studies. Regarding the cellular production and autocrine action of TGFß isoforms on cell line proliferation, such a scenario is very unlikely as huge amounts of TGFß would have to be produced within the 24h stimulation period, to reach significant concentrations in the cell culture medium. Thus, the reason for the missing effect of TGFß isoforms on the proliferation of αT3-1 and TtT/GF cells is not clear and needs to be explored in future studies.

Both SB431542 and SIS3 dose dependently and effectively inhibited the proliferation of the 2 cell lines. The growth suppressive effect was observed in the absence and presence of TGFß 1/- ß3 and no significant difference was observed in the growth inhibitory potential between untreated and TGFß treated cells. SB431542 and SIS3 are highly specific inhibitors of TGFßRI and Smad3, respectively, and no effects on other cellular signaling cascades have been reported so far. Therefore, the suppressive effects of these compounds in the absence of TGFß isoforms suggest, that the TGFß pathway may constitutively be activated in αT3-1 and TtT/GF cells. This

would then also explain why TGFß had no effect on the growth of the cell lines. Constitutive activation of intracellular signaling cascades has repeatedly been reported in pituitary adenomas but mostly in context with uncontrolled hormone hypersecretion e.g. in gsp-oncogene positive somatotroph pituitary tumor cells [165]. More detailed molecular studies are needed to approve the hypothesis of a constitutive active TGFß pathway in the murine cell lines.

In human pituitary adenoma cell cultures, the action of TGFß-1/ -3 and the two inhibitors was much more complex and different among the various tumor studies. In one tumor TGFß-1, but not TGFß-3, inhibited the growth of the cells and correspondingly, both inhibitors reverted the inhibitory action of TGFß-1 and stimulated the proliferation of the tumor cells. The stimulatory effect of the inhibitors on cell proliferation was also seen in the absence of TGFß isoforms suggesting at least a partial activation of the intrinsic TGFß system in this tumor. In two tumors TGFß-1 enhanced adenoma cell growth but the effect was not significant (this was also the case in one of the tumors, in which TGFß-3 could be tested). In these tumors both SB431542 and SIS3 inhibited cell growth in the absence and presence of TGFß isoforms. In 3 human tumor cell cultures, TGFß-1 stimulated the proliferation of the adenoma cells and the two inhibitors suppressed the growth both in the absence and presence of TGFß-1. Unfortunately, due to the limited number of adenoma cells, no studies with TGFß-3 could be performed in the tumors which responded to TGFß-1 and future studies are therefore necessary to clarify whether TGFß-3 has also proliferative activities in TGFß-1 responsive tumors. Although much more tumors need to be studied to draw final conclusions, my preliminary data suggest that in some NFPAs, probably in those that are still in a state in which they have not yet undergone EMT, TGFß-1 inhibits the growth of the epithelial tumor cells and consequently, the herein tested TGFß inhibitors would revert the inhibitory action of TGFß-1 and would stimulate tumor cell growth. During EMT, an increasing number of NFPA cells changes from the epithelial to the mesenchymal tumor type and the latter cell type may dominate in NFPAs after the process of EMT has been finished. Dependent on the state of EMT progression, cell cultures from these NFPAs may become less responsive to the inhibitory action of TGFß-1 and increasing proportions of mesenchymal tumor cells may be stimulated by this growth factor. Correspondingly, the TGFß inhibitors will act suppressive on NFPA cell proliferation only in the state, in which TGFß stimulates cell growth. To approve this speculation, much more work is necessary. In particular, using

specific markers to detect epithelial and mesenchymal tumor cells, the cellular composition of NFPAs should be characterized and it should be tested whether inhibitory or stimulatory TGFß growth effects correspond with epithelial-mesenchymal composition of the tumors. The aim of such studies should be to demonstrate that the application of TGFß inhibitors has only tumor growth suppressive action when the majority of epithelial tumor cells have changed to mesenchymal tumor cells.

4.1.3 Influence of TGFß and its inhibitors on VEGF-production

In addition to their direct effects on cell proliferation, TGFß isoforms and their receptors may influence the growth of NFPAs indirectly, through suppressing intratumoral vascularization. Beyond a size of 1 to 2 mm in diameter, cells within any solid tumor can no longer be supplied with nutrients and oxygen through diffusion but need an intrinsic blood system for their supply with these compounds. Intratumoral hypoxia is the main trigger for the development of tumor vascularization through angiogenesis. The decline of O_2 leads to the up-regulation of hypoxia-inducible factor-1, a transcription factor that induces the production of multiple factors needed for the formation of intratumoral blood vessels among them VEGF [166], playing a key role in survival of most proliferating tumor cells due to its proangiogenic and immune-modulating effects [142].

Few years ago, it has already been shown that TtT-GF cells expressed VEGF [15] and that TGFß-1 and - 3 could stimulate the production and release of VEGF [141], but at that time the TGFß inhibitors, whose action was tested in the present thesis, had not been available. Therefore, I have repeated the experiments with TGFß isoforms and could confirm the stimulatory action of both TGFß-1 and –3 on VEGF production in TtT/GF cells and moreover, could show that SB431542 and SIS3 could block the VEGF stimulatory action of the TGFß isoforms. In αT3-1 cells, I could confirm previous findings that VEGF is produced in αT3-1 cells but whereas a series of growth factors and hormones had previously been shown to have no influence on VEGF release [167], I could demonstrate in the present thesis that TGFß-1 and TGFß-3 significantly stimulate VEGF secretion in αT3-1 cells. The two TGFß inhibitors suppressed the TGFß induced VEGF production in αT3-1 cells but could also inhibit the basal VEGF release in the absence of TGFß. Thus, similar as already observed for the influence on the proliferation of the two cell lines, also the VEGF production is suppressed by the TGFß inhibitors in the absence of externally applied TGFß-1 and TGFß-3

suggesting an intrinsic activation of the TGFß pathway in αT3-1 and TtT/GF cells also in context with VEGF production. Concerning the effects of TGFß- isoforms on the proliferation and VEGF secretion, it is evident that there is a difference in the response to TGFß: whereas the latter had no influence on cell proliferation, TGFß-1 and TGFß-3 significantly stimulated VEGF production. This questions previous speculations about an intrinsically active TGFß system in the two cell lines as it is evident that at least regarding the VEGF production the intrinsic TGFß pathway is not fully activated and can therefore be further enhance by externally applied TGFß. Alternatively, the discrepancy between the action of TGFß on proliferation and VEGF production may be a consequence of the different cell culture conditions. Whereas the effect of TGFß isoforms on cell growth was studied in serum-supplied (10% FCS), proliferating αT3-1 and TtT-GF cells, investigations of the effect on VEGF production were performed under serum-reduced (0.5% FCS) conditions in growth-arrested tumor cells. The latter was necessary to exclude that effects of TGFß and the inhibitors on VEGF secretion were caused by alterations of cell numbers in proliferating cell cultures and currently it cannot be excluded that the growth arrest has effects on the cells with respect to their sensitivity to external TGFß stimulation. However, much more work is necessary to clarify this.

Unfortunately, due to the limited human NFPA tissue available, it was not possible to study the influence of TGFß isoforms and their inhibitors on VEGF secretion in primary cell cultures of NFPA. VEGF-production of hormone-secreting cells has already been shown before and it was reported that VEGF may not only play a role in intratumoral angiogenesis but may probably act as a growth factor for pituitary tumor cells [168]. Thus, the suppression of TGFß induced VEGF production by TGFß inhibitors would not only suppress tumor neovascularization but also VEGF-mediated tumor cell proliferation. As TGFß and TGFß inhibitors affected VEGF production in TtT/GF and in αT3-1 cells, I would speculate that this is the case also in human pituitary adenomas. It would be interesting - and this would have to be elucidated in future studies - whether TGFß shows opposite actions also on VEGF production in epithelial and mesenchymal pituitary tumor cells. In pituitary adenomas, intratumoral VEGF expression and vascularization starts already in microadenomas (tumor size < 1cm in diameter) [168], and progresses with increasing size and EMT in invasively growing macroadenomas. It would be interesting to see, whether at the epithelial tumor state TGFß is not only slowing down tumor development by directly inhibiting adenoma cell proliferation but

also by suppressing VEGF production and thus, intratumoral vessel formation. Conversely, at mesenchymal state after EMT, TGFß might then stimulate VEGF mediated vascularization. This would strengthen the argument that for the application of TGFß inhibitors as pharmacological inhibitors of pituitary adenoma development the epithelial state of the tumors would have to be determined to avoid adverse stimulatory effects of the inhibitors at early tumor development stages. However, much more work in primary cell cultures of pituitary tumors is needed to clarify all these speculations.

4.2 General discussion

4.2.1 Pituitary tumor development and EMT

Tumor formation implies the structural reorganization of neoplastic cells, in order to enable its survival and progression, independent from the surrounding healthy tissue and to protect it from the immune system. Thus, cells with distinct properties and tasks develop during neoplastic transformation and tumor progression. There are cancer stem cells, differentiated cancer cells, cancer-associated fibroblasts and endothelial cells [169]. Noncancer stromal cells and ECM constitute the tumor microenvironment [169]. Adenoma/ carcinoma derive from epithelial cells. The transformation of these epithelial cells into mesenchymal cells is an essential step in tumor formation/ progression and metastasis (type-2 EMT).

Epithelial-mesenchymal-transition (EMT) describes a gradual phenotypic shift from epithelial cells, characterized by tight cell-cell-junctions and clear polarity (basal and apical), into spindle-like fusiform motile mesenchymal cells [126]. On a molecular level, it is characterized by the loss of epithelial-cadherin (E-cadherin) and the gain of mesenchymal markers such as neural-cadherin (N-cadherin) and Vimentin [72]. Besides, EMT leads to an enhanced expression of transcription factor snail family transcriptional repressor 1 (also referred to as Snail), the transcription factor snail family transcriptional repressor 2 (SNAI2) (also called Slug), forkhead box C1 (FOXC1) and twist-related protein 1 (TWIST1) [72].

EMT is an essential process towards invasiveness and it was shown that the epithelial marker E-cadherin is significantly lower expressed in invasive PA as compared to their noninvasive counterparts [72].A positive correlation for high expression of Slug, ERα and invasiveness of pituitary adenoma could also be demonstrated, highlighting EMT as an essential key to invasive behavior of pituitary adenoma [72]. Promoting/initiating

signaling pathways and molecules are thus an important therapeutic target against invasive and aggressive PA or to stop progression of borderline tumors.

TGFß is a known potent inducer of epithelial plasticity, by combining both Smad-dependent- and Smad-independent signaling [169]. Smad3/4, for instance, induces the expression of Snail by directly binding of the regulatory promotor sequences [169] and the E3 ubiquitin-ligase HDM2. HDM2 leads to ubiquitylation and degradation of p53, which correlates with EMT progression [169]. TGFß is also involved in the expression of MMPs such as MMP2 and MMP9, fibronectin and collagens [169]. However, alternate intracellular signaling pathways activated by TGFß were shown to promote EMT in pituitary adenoma progression, for instance the PI3K/Akt/mTOR pathway [169]. Those non-Smad signaling pathways were not examined as part of this theme, but have to be taken into account for the interpretation of our results.

Still, TGFß also has inhibitory function against epithelial proliferation, consequently acting as a tumor suppressor and against mesenchymal transformation. Genetic alterations in TGFß-signaling components and subsequent pathways may inactivate TGFß´s growth inhibitory properties. This might shift the balance, making TGFß in turn an essential factor promoting tumor progression, for instance via EMT [169]. Increased levels of TGFß, or overexpression of the TGFβ- receptors by cancer cells result in increased autocrine stimulation/activation in the tumor microenvironment. As a result, cancer cells might be able to use TGFß-signaling as a key to survive and form metastasis [169]. For instance, the breast cancer model cell-line MDA-MB-231, that expresses TGFß-receptors and does secrete TGFß was shown to be resistant to the growth inhibitory function of TGFß, but responsive to TGFß with an increase in spreading and invasiveness [170].

The dual role of TGFß in tumorigenesis described by other authors corresponds to our results with two human NFPA-samples, as the external application of both TGFß-1 and TGFß-3brought contrary results in regard to cell proliferation. In NFPA1 TGFß-1/3 inhibited, while in NFPA2 TGFß-2/-3 stimulated proliferation. Correspondingly, in NFPA1, in which TGFß inhibited cell growth, the application of the Smad3 inhibitor SIS3 and of the receptor inhibitor SB431542 stimulated adenoma cell proliferation, while in NFPA2 these inhibitors suppressed proliferation. Thus, the tumors tested do likely differ in their stage, characterized by a switch of TGFß from inhibitory to stimulatory function/ effect on cell proliferation. Both tumor-types must have lost though, their sensitivity to external application of TGFß in either direction, as inhibiting

or promoting agent for proliferation. Increasing evidence by other authors suggest, that after cells lose their sensitivity to TGFß-mediated growth inhibition, autocrine TGFß-signaling may then promote TGFß-signaling [170]. Several studies could also show that expression of a dominant-negative TGFßRII, which is thus unresponsive to TGFß stimulation, can prevent the conversion of cells from an epithelial to an invasive mesenchymal phenotype, delay tumor growth and reduce metastasis. This underlines the importance of autocrine TGFß-signaling in tumor progression [170]. However, constitutively active TGFβI-receptors as the downstream components of the receptor complex, that can signal independent from TGFβRII and ligand, have been described by various authors [171].

In our experiments with the mouse cell models, external application of TGFß-1 and TGFß-3alone showed only limited, not significant effect on cell proliferation. This accounted for both αT3-1 and TtT-GF-cells. SB431542 and SIS3 however dose-dependently inhibit αT3-1 and TtT-GF-cell proliferation both in the presence and absence of TGFß-1 and TGFß-3, as was observed repeatedly, most significantly using ^{3}H-thymidine Incorporation assay. This might either suggest a constitutive active Smad signaling pathway in respect to cell-proliferation, or to an endogenous production of TGFß-1/3 and autocrine stimulation. The relevance of autocrine TGFß-signaling in tumor progression have already been demonstrated by several authors [170]. In a study by Ghosh et al. from 2008 PPAR-γ null-mouse embryonic fibroblasts Smad 2/3 were constitutively phosphorylated and predominantly located in the nucleus. This activation of Smads was ascribed to increased levels of TGFß and TGFβRI kinase, as the ALK5 inhibitor SB431542, which we also used for our experiments, blocked phosphorylation and transfer into the nucleus [172]. In our experiments, the external application of TGFß led to a significant increase in cell proliferation. SB431542 and SIS3 blocked cell proliferation, both in the absence and presence of TGFß. The fact that SB431542 and SIS3 also led to a significant decrease in cell viability without external application of TGFß suggests that the signaling pathway is activated, at least in part, due to autocrine stimulation or constitutively active components of the signaling pathway.

It has to be taken into account, that the inhibitors we used in our experiments do solely interfere in Smad-dependent signaling. SB-431542 is a specific inhibitor of TGFβ superfamily type I receptors ALK4, ALK5 and ALK7 [137], which doesn´t interfere with other intracellular pathways activated by TGFβ, such as the ERK, JNK, or MAPK

pathways as was shown by other authors [137]. SIS3 is a selective inhibitor of Smad3-phosphorylation [65]. The role of SB-431542 was dual dependent upon the respective tumor-cell-line, as was suggested by previous findings by other authors [137]. Our findings suggest that it might more likely also depend upon the stage of the respective tumor. Consequently, SB431542 might be promising in later stages of tumor development as a therapeutic agent, but can be worsening in earlier stages. Further studies will be needed to accurately predict the stage in which SB431542 might be a useful anti-tumor therapeutic agent.

4.2.2 VEGF and vascularization in pituitary tumors

Ensuring adequate blood supply is essential for survival of neoplastic cells and tumor growth, since otherwise they might suffer from hypoxia, leading to necrosis [142]. For this, augmented angiogenesis via an autonomous, autocrine secretion of pro-angiogenetic factors is an important feature seen in various tumors [142]. Although VEGF overexpression was generally reported in pituitary adenoma by various authors [146], this observation did not correlate with microvascular density of those tumors. The majority of pituitary adenoma have low MVD in contrast to their high VEGF-expression pattern [146]. This also applied for invasive pituitary adenoma, as was demonstrated by various studies [146]. However, in a study including 157 pituitary adenoma samples with distinct cellular origin, Vidal et al. observed that invasive pituitary tumors tended to be more highly vascularized as their non-invasive counterparts. The authors did not see a major correlation in between MIB-1 index and microvascular density [173]. Independent of their state of vascularization, VEGF/VEGFR signaling and high VEGF correlate with disease progression and hemorrhage in pituitary neuroendocrine tumors [174] and especially NFPA, macroprolactinoma and invasive/ aggressive pituitary adenoma show high VEGF-expression levels [72]. Some authors suggested, that in spite of low MVD the well-preserved expression of VEGF might contribute to adequate vascular supply with distinct complex mechanisms other than endothelial cell proliferation [151]. More importantly maybe, other non-angiogenetic influences of the VEGF/VEGFR pathway have to be taken into account as well, supporting tumor progression. Besides its promoting effect on proliferation of endothelial cells, VEGF binds to hematopoetic progenitor cells that express VEGFRI and influences development and differentiation on immune cells [142]. For instance, it has been demonstrated that VEGF inhibits the development of dendritc cells both *in vitro* and *in vivo* and has been identified as one

of the primary soluble factor produced by tumors to suppress immune function, which would compromise tumor survival [142].

Especially Prolactinoma and non-functioning pituitary adenoma show a high VEGF-expression level [148]. This accounts for invasive macroprolactinoma and NFPA, as for invasive and aggressive pituitary adenoma in general [72]. VEGF-inhibition has already been the focus of researches for alternative therapeutic agents against NFPA, mainly Cabergoline and Somatostatin-analogs, but their inhibitory function was dependent upon expression levels of the respective receptors or lack thereof [80, 81]. Whether my findings about the inhibitory action of the TGFß receptor blocker SB431542 and the Smad3 inhibitor SIS3, which both effectively suppressed basal and TGFß-1/-3 induced VEGF secretion in pituitary tumor cell lines will be the basis for the development of a new pharmacological concept for the treatment of NFPA and probably other types of pituitary tumors, in particular at advanced stages, needs to be clarified in future studies.

4.3 Limitation of the study

There are some limitations of this study. One of them is the fact that I had to focus my investigations on pituitary tumor cell lines which is however often seen in pituitary tumor research, as the amount of tissue obtained from human pituitary tumors is small and often insufficient for big series of experiments in primary cell culture. It is also not possible to propagate cells from human pituitary tumors and to cultivate them for longer time periods. These problems have to be overcome by the use of cell lines. Hereby the gonadotroph αT3-1 cell is an established model for gonadotroph-derived nonfunctioning pituitary tumors, which represent the vast majority of NFPAs. In contrast, the use of the folliculo-stellate-like TtT/GF cells as a model for NFPAs is critical. Folliculo-stellate cells, however, make up an essential part of tumor microenvironment as has been confirmed for various pituitary adenoma subtypes [175]. Several reports describe a high plasticity of TtT/GF cells and it has been shown that they have properties of immune cells, stem cells, endothelial cells and pericytes suggesting an unclear origin and function of this multifaceted cell line. Interestingly, very recently it has been shown that long-term treatment of TtT/GF cells with TGFß-2, a TGFß isoform that was not used in the present thesis, induced a pericyte phenotype in this cell line, underlining the unclear nature of this cell type [176]. The main limitation of this study is the paucity of tumor samples in which the findings from the cell lines

should be approved. During the approximately 6 months lasting experimental part of my thesis finally only 6 human pituitary adenomas could be used for my studies and as only very few cells could be obtained after tissue dispersion only limited growth experiments could be performed and no material was left to study VEGF secretion. Moreover, the cellular origin and the epithelial/mesenchymal state of the pituitary tumors according to the WHO classification [10] was not evaluated, as this is not part of routine pathology, in which at best the absence of hormone expression is studied. As all tumor material received from the neurosurgical centers was used completely for the growth studies, no tissue was left to perform corresponding pathological studies myself. Thus, I have no possibility to compare the different effects of TGFß on NFPA cell proliferation with the origin or state of the NFPAs. However, as waiting for additional NFPAs would have by far exceeded the time frame of my thesis, there was no possibility to increase the number of human tumors studied and only the present very preliminary data can be presented. Much more tumors need to be investigated in future studies to get comprehensive information about the role of TGFß in NFPAs and the relevance of TGFß inhibitors as future pharmacological treatment options of these tumors.

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Affidavit

Eidesstattliche Versicherung

van Saan, Marie Céline

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

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